Biochemical studies on blackspot bruising in potato (solanum tuberosum l.)

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Biochemical Studies on Blackspot Bruising in Potato (*Solanum tuberosum* L.)

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For my parents, and for Beth

ABSTRACT

Potatoes respond to mechanical stress by invoking a precisely controlled biochemical reaction leading to production of the pigment melanin. Predisposition to produce melanin is variety specific, this specificity being conveyed by a range of genetic, environmental and biochemical factors.

A well characterised response of plant tissues to perturbation by biotic or abiotic stress factors is the rapid generation of oxygen based free radicals – active oxygen species (AOS). Due to their nature as high-energy, largely unstable, radicals, AOS have been implicated in a wide range of deleterious effects upon plant tissues as well as being involved in a range of signalling cascades within cells.

This project attempted to demonstrate a link between AOS and other associated oxidative factors, and blackspot bruise potential.

It was demonstrated that in response to mechanical impact, potato tubers respond by producing a well-defined level of superoxide free radicals. The amount of superoxide generation was found to be nearly perfectly correlated with susceptibility to blackspot bruise when investigated over eight potato varieties. By using inhibitors and scavengers of free radicals it was shown that superoxide and not hydrogen peroxide was directly responsible for the effects upon pigment synthesis. Furthermore studies upon polyphenol oxidase (PPO), the key enzyme in the synthesis of melanin, demonstrated that a significant enhancement of activity was noted in the presence of superoxide radicals, lending support to animal studies which suggest that PPO utilises superoxide radicals more readily and more efficiently than its usual co-substrate molecular oxygen.

Associated with the production of superoxide radicals was the demonstration of modification to tuber proteins, specifically the detection of secondary carbonyl groups, a modification known to be the direct result of exposure to AOS. This study quantified the carbonyl levels and once again these were almost perfectly correlated with susceptibility to blackspot bruise formation.

Once the correlation of oxidative factors and blackspot bruise susceptibility was established then studies were undertaken to demonstrate the effect of a range of metal ions upon both blackspot bruise susceptibility and oxidative protein modifications. These studies suggested that potassium and zinc both had negative effects on blackspot susceptibility however only potassium had a negative effect on secondary carbonyl accumulation.

Studies on tubers impacted twice indicated that the normal 'polarity' of the tuber in terms of susceptibility to blackspot bruise could be influenced and this was confirmed by studies on superoxide generation which showed that the second point of impact always had a reduced blackspot susceptibility and reduced superoxide generation.

Finally a model is presented to incorporate this novel information regarding the role of AOS in blackspot bruise susceptibility together with existing knowledge of the biochemistry within this system, to propose a system in which AOS play an integral and wide-ranging role.

DECLARATION

I confirm that no part of the material presented has previously been submitted for a degree in this or in any other university. If material has been generated through joint work, my independent contribution has been clearly indicated. In all other cases material from the work of others has been acknowledged and quotations and paraphrases suitably indicated.

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ABBREVIATIONS

AC	Adenylate cyclase
AIDS	Acquired immunodeficiency syndrome
AOS	Active oxygen species
BSA	Bovine serum albumin
CAT	Catalase
CIP	Centro Internacional de la Papa
cv.	Cultivar
CWP	Cell wall peroxidase
Cyt	Cytochrome
DHI	5,6-dihydroxyindole
DHICA	5,6-dihydroxyindole-2-carboxylic acid
DNP-	Dinitrophenyl-
DNPH	2,4-Dinitrophenylhydrazine
DOPA	Dihydroxyphenylalanine
DPI	Diphenylene iodonium chloride
E	Elicitor
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
G	G-protein
GA64	Glu : Ala (6:4) synthetic peptide
GAT111	Glu : Ala : Tyr (1:1:1) synthetic peptide
GAT631	Glu : Ala : Tyr (6:3:1) synthetic peptide
GTP	Guanidine tri-phosphate
HEPES	*N*-(2-Hydroxyethyl)piperazine-*N*'-(2-ethanesulfonic acid)

HRI	Horticulture Research International
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCD	Programmed cell death
PLase	Phospholipase
POD	Peroxidase
PPO	Polyphenol oxidase
PVY	Potato virus Y
ROS	Reactive oxygen species
SBEU	Sutton Bridge Experimental Unit
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
TCA	Tri-chloro acetic acid
TFA	Tri-fluoro acetic acid
XTT	2,3-*bis*(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

Trading Names

Coomassie-Blue R	$C_{45}H_{44}N_3O_7S_2Na$
Tris	Tris(hydroxymethyl)aminomethane
Tween 20	Polyoxyethylenesorbitan monolaurate

PUBLICATIONS AND PRESENTATIONS

Published Work

Croy, R.R.D., Baxter, R., Deakin, W., Edwards, R., Gatehouse, J.A., Gates, P., Harris, N., Hole, C., Johnson, S.M. and Raemaekers, R. (1998) Blackspot bruising in potatoes: structural and molecular approaches to the identification of factors associated with tuber bruising susceptibility. *Asp App Biol* **52** 207-214

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Conference presentations

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INTRODUCTION

1.1 Introduction

1.1.1 Overview

Potato (*Solanum tuberosum)* is a member of the Solanaceae, an economically important and diverse family of plants, examples of which are listed in table 1.1. Solanaceae family members include medicinally valuable plants such as *Atropa bella-donna* (Deadly Nightshade), from which the anticholinergic drug atropine is obtained, and *Hyoscyamus niger* (Henbane), from which the mild sedative and 'truth drugs' hyoscine and scopolamine are obtained. The family also includes horticulturally valuable plants such as Winter Cherry and Jerusalem Cherry. However, the solanaceae is predominantly recognized for the three internationally valuable crops; potato, tomato and tobacco.

The potato is believed to have been first cultivated in Chile, Peru and Bolivia by at least AD200 as it is regularly seen as a motif on Indian pottery from these areas at this time. It has been suggested that the potato first came to Europe by way of a Spanish monk who brought examples back to Spain in the 16th century, from where it passed to Italy, France and Belgium. Sir Francis Drake is believed to have brought potatoes back to England from South America in 1563 but this was doubted by Sir Joseph Banks, who in the early 19th century suggested they had brought back the sweet-potato *Ipomoea batatas*, which is not a member of the Solanaceae. However Banks' suggestions have recently largely been discounted. Sir Walter Raleigh definitely did bring the potato to his Cork estate in 1586, however the plant was already being grown in continental Europe at this time (Brimble, 1957)

Table 1.1 Examples of species of the Solanaceae . Table shows the diversity of the family, including foods (potato, tomato) ; drugs (deadly nightshade) ; horticultural (winter cherry)

Common Name	Latin Name
Potato	*Solanum tuberosum*
Tomato	*Lycopersicon esculentum*
Tobacco	*Nicotiana tabacum*
Deadly Nightshade	*Atropa bella-donna*
Henbane	*Hyoscyamus niger*
Bittersweet	*Solanum dulcamara*
Winter Cherry	*Solanum capsicastrum*
Potato Vine	*Solanum jasminoides*
Jerusalem Cherry	*Solanum pseudocapsicum*

Historically the most significant event related to potatoes of recent times was the 'Irish Potato Famine' of 1846-1851 when the fungal pathogen *Phytophthora infestans* (Potato late blight fungus) caused almost total loss of the Irish potato harvest. During the ensuing famine almost 20% of the Irish population emigrated, predominantly to the United States. In more recent times, significant losses of potato harvests have been caused by *Leptinotarsa decemlineata* (Colorado potato beetle), which is a native of North America but has spread to Europe. It is of sufficient danger to UK harvests that sightings in the UK of the Colorado potato beetle must be notified to the police. Other potato pests include the potato leafhopper and the potato flea beetle, both of which have the potential to cause significant losses to potato harvests. Despite intensive breeding programmes and development of genetically modified varieties many types of micro-organisms also pose threats to potato plants, including bacteria e.g. soft rot (*Erwinia carotovora)*, viruses e.g. P.V.Y. and fungi e.g. late blight (*Phytophthora infestans)*.

Potatoes have come to occupy a invaluable role to both producers and consumers worldwide (see Table 1.2 for a guide to the economic importance of potatoes on a continent by continent basis, and Table 1.3 for a list of countries within each continent for which potatoes are either the first or second most economically important crop). The reason why they have assumed such global importance is due primarily to the ease with which potatoes can be grown and manipulated by breeding to select certain desirable traits. Indeed the potato is now available in a vast number of different varieties, which themselves have been derived from crosses between parental varieties in an attempt to 'collect' desirable traits into one variety. Of course, the term desirable traits has differing connotations depending upon, for example, where you are in the world. US and British markets tend to prefer lighter texture potatoes when compared to

Table 1.2 Statistics of World Potato Production. 1995-1997 production is mean over 3 years and rounded to nearest 1 000 tonnes. *Economic Importance is relative economic contribution of potato growing by comparison with the 21 other major world food crops. Data from C.I.P., Peru

Continent	Production 1995-97 (Tonnes)	% of World Production	Economic Importance *
ASIA & OCEANIA	83 120 000	28	5
AFRICA	7 692 000	3	10
SOUTH/CENTRAL AMERICA	14 486 000	5	7
EUROPE & RUSSIA	154 430 000	52	2
NORTH AMERICA	25 113 000	9	4
WORLD	295 118 000		4

Table 1.3 Worldwide importance of potatoes. Countries for which Potatoes are of primary or secondary economic importance.

Continent	Countries for which Potato is the first or second most important economic crop		
ASIA	IRAN (2)	BANGLADESH (2)	
AFRICA	ALGERIA (2)	MALAWI (2)	
SOUTH/CENTRAL AMERICA	COLOMBIA (2) CUBA (1)	PERU (1)	BOLIVIA (2)
EUROPE	RUSSIA (1) NETHERLANDS (1)	POLAND (1)	UKRAINE (1)

mainland Europe where heavier textured tubers are preferred. Certain characteristics, such as the reduction of disease susceptibility, are universal, however.

1.1.2 Structure

Potato tubers, the generally subterranean, edible, starch-storing organs, are a form of modified stem tissue, correctly termed a stem-tuber. Branch stems, from the axillary buds of base leaves, grow underground, swelling at their tips to form tubers. An essential part of potato growing is the drawing of soil up over the lower leaves of the plant - this helps promote the lateral growth from the axillary buds. The potato tuber thus has an internal structure similar to that of the stem, including the arrangement of xylem and phloem (Brimble, 1957). The purpose of the tuber to the potato plant is twofold, first as a vegetative reproduction body - hence the presence of spirally arranged buds ('eyes' – the rose end) from which shoots can form under appropriate conditions after a period of dormancy (the majority of potatoes are grown from 'old' tubers).

Secondly the tuber plays a role as a food store, containing large reserves of starch, indeed up to 80% of the organic material in potatoes can be starch, generated by the action of sucrose synthase upon imported sucrose. The starch can be called upon when required, and, after initial action by an endoamylase, the action of phosphorylase and debranching enzymes leads to the production of glucose-1-phosphate, which cycles through UDP-glucose and fructose-6-phosphate leading ultimately to the production of sucrose for export to the new plant. (Brimble, 1957 ; Sowokinos, 2001).

1.1.3 Economic Importance

Potatoes are of very significant economic importance throughout the world. Recent figures published by the Centro Internacional de la Papa (CIP), Peru indicate that potatoes are grown on all continents of the world, except Antarctica, and economically are the 4th most important crop worldwide (Table 1.2).

Of the figures presented in Table 1.2 for European potato production (154 million tonnes) the United Kingdom produced 6 171 000 tonnes per year over the period 1995-1997. This represents 2% of the world potato production, and 4% of the European production. This tonnage was produced by approximately 12 400 registered producers (British Potato Council data). Over the period in question the average price per tonne paid to potato producers was a little over £120, which means that the mean UK potato crop value in the period 1995-1997 was approximately £741 million per year to the potato producers.

1.2 Blackspot Bruising

1.2.1 Overview

Being a large, heavy vegetable with a high water content, potatoes are especially sensitive to physical damage, particularly as they grow underground and must be mechanically harvested. Physical damage to potatoes includes slicing and piercing by harvesting equipment, as well as mechanical impact damage – caused when tubers suffer a pressure trauma either by falling onto hard surfaces, jostling or bumping against each other. Mechanical impact damage is predominantly caused during harvesting when tubers fall from harvesting machinery into trailers, or onto each other, however all stages of handling subsequent to harvesting can also cause mechanical

impact damage. Slicing and piercing injuries have obvious consequences when it comes to grading for consumption. Mechanical impact damage, however, causes a chain of biochemical events to be initiated, in which the blue-black pigment melanin is produced subdermally in the vicinity of the impact. The subdermal region of pigmentation caused by mechanical impact damage is known variously as a blackspot bruise or bluespot bruise (Horne, 1912), for the purposes of this thesis I shall refer to this phenomenon as 'blackspot bruise' or simply as 'bruising' which are the more widely used terms at this time. The biochemistry of blackspot bruise is treated in section 1.3, however in summary, when tubers are mechanically impacted, a level of decompartmentalisation of cellular contents takes place. This mixing of cytosolic components with vacuolar components leads to the enzyme polyphenol oxidase (PPO, tyrosinase, EC1.14.18.1) encountering monophenolic substrates, predominantly the amino acid tyrosine. PPO catalyses the formation of various di-phenols (e.g. dihydroxyphenylalanine (DOPA)) from monophenols e.g. tyrosine. The di-phenols then undergo auto-oxidation steps to quinonic compounds, upon which PPO again acts, to form polyphenolics, of which the pigment melanin is the principal end product. The introduction of these dark coloured pigments into the tuber tissue may make them distasteful due to accumulation of quinonic and glycoalkaloid compounds leading to both bitterness and astringency (Mondy *et al.*, 1971 ; Sinden *et al.*, 1976 ; Zitnac and Filadelfi, 1985 ; Johns and Keen, 1986 ; Kaaber, 1993) but also causes significant (up to 40%) rejection of entire batches of tubers because of the undesirable visual appearance. This high level of rejection causes significant financial losses to potato producers worldwide – in Britain crops are devalued by approximately £50/tonne due to black spot bruising – where the average weekly price for potatoes is around

£120/tonne this therefore can realise up to a 45% depreciation of income to farmers who harvest a potato crop particularly susceptible to blackspot bruise.

1.2.2 Other forms of potato tuber mechanical impact damage

Although blackspot bruise, characterised by membrane damage within cells, is the typical response to mechanical stress encountered by potato processors, other forms of mechanical impact damage occur. It has been demonstrated that when drop heights for tubers exceed ~38cm, blackspot bruise does not take place to any significant degree, and other, more severe, forms of damage predominate (Mathew, 1992). These, more severe, forms of damage include cell wall failure (which is not required for blackspot bruise), cracking between cells and ultimately a combination of cracking, cell wall and cell membrane failure (widely known as shatter bruise). It appears that as drop heights increase the incidence of blackspot bruise initially increases before decreasing as shatter bruise incidence increases (Mathew, 1992).

1.2.3 Variability

1.2.3.1 Environmental and physical effects on bruise development

Environmental factors affecting tuber variability to blackspot bruise are numerous however only those of principal interest to the growing industry are discussed here having been extensively studied by agronomists.

Research concerned with the energy of impact and shape of impacting body upon tubers has shown that a spherical impacting body doubled both the volume and depth of tuber bruise when compared to impact (at the same energy) against a flat faced body, though the radius of curvature factor was found to be largely irrelevant. Further when

the impact energy was decreased by 10% the tubers showed approximately 95% reduction in bruise volume and depth, interestingly the authors suggested that an impact energy as little as 0.067J was sufficient to initiate bruising responses – this being equivalent to an average medium sized tuber falling only 3cm onto another tuber (Molema *et al.*, 1997)

The use of mineral nutrition to avoid bruise susceptibility is one of the oldest agricultural strategies in this area. The key enzyme associated with the process, PPO (section 1.3.3), contains copper as a cofactor and thus copper has been extensively investigated as a factor influencing blackspot (Lerner & Fitzpatrick, 1950 ; Kertesz, 1952 ; Mulder, 1956), however due to the very low requirement by plants for copper the effect of this mineral deficiency has been deemed to be of negligible importance – work in the 1950's did however indicate that tubers grown on copper deficient media were more resistant to blackspot bruise (Mulder, 1956).

Although no mechanism has been proposed, zinc has a reported negative effect upon the synthesis of tyrosine, the PPO substrate, and thus zinc in the form of fertilizers has been used to control blackspot bruise – however a loss of plant vigour and toxicity due to zinc excess has led to its withdrawl (Mondy & Chaudra, 1981).

One mineral which has, however, been consistently associated with blackspot bruise resistance is potassium. Potassium, is known to reduce levels of tyrosine in potato tissues (Mulder, 1949 & 1956 ; Mondy *et al.*, 1967), and may also have a negative effect (perhaps indirectly) upon PPO levels (Mondy *et al.*, 1967 ; Birecki *et al.*, 1971 ;

Matheis, 1987a ; McNabnay *et al.,* 2000). The mineral is currently widely used in growing programmes and by agriculturalists to induce blackspot bruise resistant crops.

Storage of tubers increases their susceptibility to blackspot bruise. Increased maturity is known to increase the levels of the substrate tyrosine and thus increases the blackspot bruising potential (Mapson *et al.,* 1963). Associated with tuber maturity is tuber specific gravity, with increasing tuber maturity, specific gravity increases and a positive correlation of R=0.933 between specific gravity and bruising potential has been observed in a single variety (Massey *et al.,* 1952 ; Cole, 1975 ; Killick & MacArthur, 1980).

Table 1.4 summarises these, and other, environmental factors, known to have effects on blackspot bruising.

1.2.3.2 Genetic Factors

The potato is one of the most extensively cultivated plants in the world. Very extensive breeding programmes to introduce desirable character traits have led to a very wide range of available cultivars with large numbers of cultivars now having been discontinued and superceded by newer and 'better' cultivars. As a consequence the handbook of potato varieties produced by the British Potato Council lists no less than 29 variables (table 1.5) for each tuber variety ranging from drought tolerance and tuber size through to leaf roll virus resistance and cooking discolouration factors. Within blackspot bruise resistance factors there is substantial variation; for example amongst those crops grown widely in the UK – Maris Piper and Cara both exhibit high bruise resistance whereas Russet Burbank and Navan have high bruise susceptibility. A Table

Table 1.4 : **Environmental factors affecting blackspot bruise susceptibility.** Compiled from data presented in Brook, 1995

FACTOR	Effect upon blackspot susceptibility	Suggested mode of action	Reference
Copper (Increased)	Increase (slight)	Enzyme cofactor	Mulder, 1956
Zinc (Increased)	Reduce	Reduced tyrosine synthesis	Monday & Chaudra, 1981
Potassium (Increased)	Reduce	Reduced tyrosine synthesis	Monday *et al.*, 1967 ; Mulder, 1949 ; Mulder, 1956
Tuber maturity	Increase	Increased tyrosine	Mapson *et al.*, 1963
Specific Gravity (Incr)	Increase	Increased maturity	Massey *et al.*, 1952 ; Cole, 1975 ; Killick & MacArthur, 1980
Bigger tubers	Increase	Greater impact energy	Hughes, 1980
Larger tuber cell size	Increase	Cells more elastic	Reeve *et al.*, 1973 ; Hudson, 1975 ; VanEs & Hartmans, 1975
Delaying harvesting	Increase	Increased maturity	Brook, 1996
High dry matter content	Increase	Greater damage due to starch grains	Ophius *et al.*, 1958
Ethylene	Reduce	Callus induction	Timm *et al.*, 1976
Long term storage	Increase	Phenolic level change	Mapson *et al.*, 1963

Table 1.5 : Characteristics listed for British Potato cultivars. From Dixon, 1992

No.	Variable	Range
1	Dormancy Period	Short – Long
2	Foliage Maturity	Late – Early
3	Drought Tolerance	Low – High
4	Tuber Shape	Round – Oval – Long Oval
5	Eye Depth	Very Deep – Very Shallow
6	Flesh Colour	White – Cream – Deep Yellow
7	Skin Colour	White – Yellow – Red – Blue – Mixed
8	Early Yield	Low – High
9	Total Mature Yield	Low Marketable – High Marketable
10	Tuber Size	Small – Large
11	Tuber Number	Low – High (per plant)
12	Freedom From Outgrades	Low Freedom – High Freedom
13	Damage (Splitting Resistance)	Poor – Good
14	Blackspot Bruise	Susceptible – Resistant
15	Foliage Blight Resistance	Low – High
16	Tuber Blight Resistance	Low – High
17	Blackleg Resistance	Low – High
18	Common Scab Resistance	Low – High
19	Powdery Scab Resistance	Low – High
20	Gangrene Resistance	Low – High
21	Potato Leaf Roll Virus Resistance	Low – High
22	Potato Virus Y Resistance	Low – High
23	Spraing Resistance	Low – High
24	Slug Attack Resistance	Low – High
25	PCN (RO_1)	Resistant – Partial Resistance – Susceptible
26	Dry Matter	Low – High
27	Fry Colour	Poor – Optimum
28	Discolouration (Cooked)	Discoloured – No Discolouration
29	Disintegration (Boiled)	Disinegrated – Intact

showing the bruise susceptibilities of the commonly grown UK potato varieties is given in table 1.6.

1.2.4 Economic Importance

Recent figures published by the British Potato Council suggest that blackspot bruising alone causes a crop devaluation of £50 per tonne of harvested material in the UK. This devaluation is due to poor visual and taste quality, causing potato processors to reject significant quantities of tuber harvests – many industrial companies have certain thresholds of damage level: if a sample of a harvested crop fails to meet this threshold at the point of receipt from the transporter the entire shipment will usually be refused. Given this devaluation the total losses to the British potato industry due entirely to blackspot bruising was estimated in 1998 to be £26.4million. In 1994 the equivalent figure for the US potato producers was a devaluation of $298.9million (Brook, 1996). Given these figures it is obvious that blackspot bruising causes very serious financial losses to the majority of potato producing countries and research into a means to reduce blackspot bruise damage is imperative to prevent such significant financial losses on a global level.

1.3 Biochemistry of pigment formation and correlations with bruise susceptibility

1.3.1 Overview

The production of the blue-black melanin pigment is the characteristic biochemical response of potato tubers to mechanical stress (Muneta, 1977 ; Walker, 1977 ; Rhodes & Wooltorton, 1978). Mechanical stress in the form of an impact causes membrane distortion and disruption, though cell wall rupture *per se* is not required to induce melanin production (section 1.2.2). Membrane disruption causes

Table 1.6 : Blackspot bruise susceptibility of a range of common British potato varieties with a range of uses. The range of blackspot bruise susceptibility runs from 1 (highly susceptible) to 9 (highly resistant) to bruise. From Dixon, 1992

VARIETY	BLACKSPOT SUSCEPTIBILITY
Arran Pilot	7
Cara*	8
Cultra	6
Desiree	5
Estima	7
King Edward*	7
Marfona	6
Maris Peer	6
Maris Piper*	7
Nadine	8
Navan	3
Pentland Dell*	5
Record*	5
Russet Burbank*	4
Saturna	4

* - indicates varieties used in this study

decompartmentalisation of the plastid located enzyme polyphenol oxidase (PPO) [section 1.3.3] releasing the enzyme into the cell cytoplasm and mixing it with its substrates (Hughes, 1980). PPO then hydroxylates the amino acid tyrosine [section 1.3.2], located predominantly in the cytosol to dihydroxyphenylalanine (DOPA) [section 1.3.4]. The DOPA then undergoes various non-enzymatic auto-oxidation reactions leading ultimately to the production of the pigment melanin [section 1.3.6].

1.3.2 Tyrosine

Tyrosine is the principal substrate for PPO in the biochemical pathway leading to melanin-pigment based products (Lerner & Fitzpatrick, 1950 ; Stevens & Davelaar, 1997), and as a consequence is one of the main determining factors for potato tuber tissues ability to produce blackspot bruise pigments (Stark *et al.*, 1985 ; Corsini *et al.*, 1992 ; Mondy & Munshi, 1993 ; Stevens & Davelaar, 1997). Tyrosine is synthesised via the shikimate pathway, of which chorismate mutase is a key regulatory enzyme (Gilchrist & Kosuge, 1980 ; Jensen, 1986). The pathway is also that by which phenylalanine is synthesised although phenylalanine is thought to play little or no role in determining the extent of blackspot bruising (Dean *et al.*, 1992). Upon mechanical impact, membrane disruption occurs, releasing PPO from the plastids into the cytosol where tyrosine, as a monophenol, in the presence of molecular oxygen and PPO, undergoes hydroxylation to yield dihydroxyphenylalanine (DOPA) which is discussed in section 1.3.4 (Corsini *et al.*, 1992), a reaction which is substantially accelerated by the presence of Cu^{2+} ions which may act as an enzyme cofactor (Kertesz, 1952). The oxidation of tyrosine to DOPA, followed by subsequent reactions ultimately leading to melanin production was first characterised by Mason, 1955 and the pathway is known

as the 'Raper-Mason pathway' (Mondy *et al.*, 1960 ; Vertregt, 1968 ; Matheis *et al.*, 1978 ; Stark *et al.*, 1985).

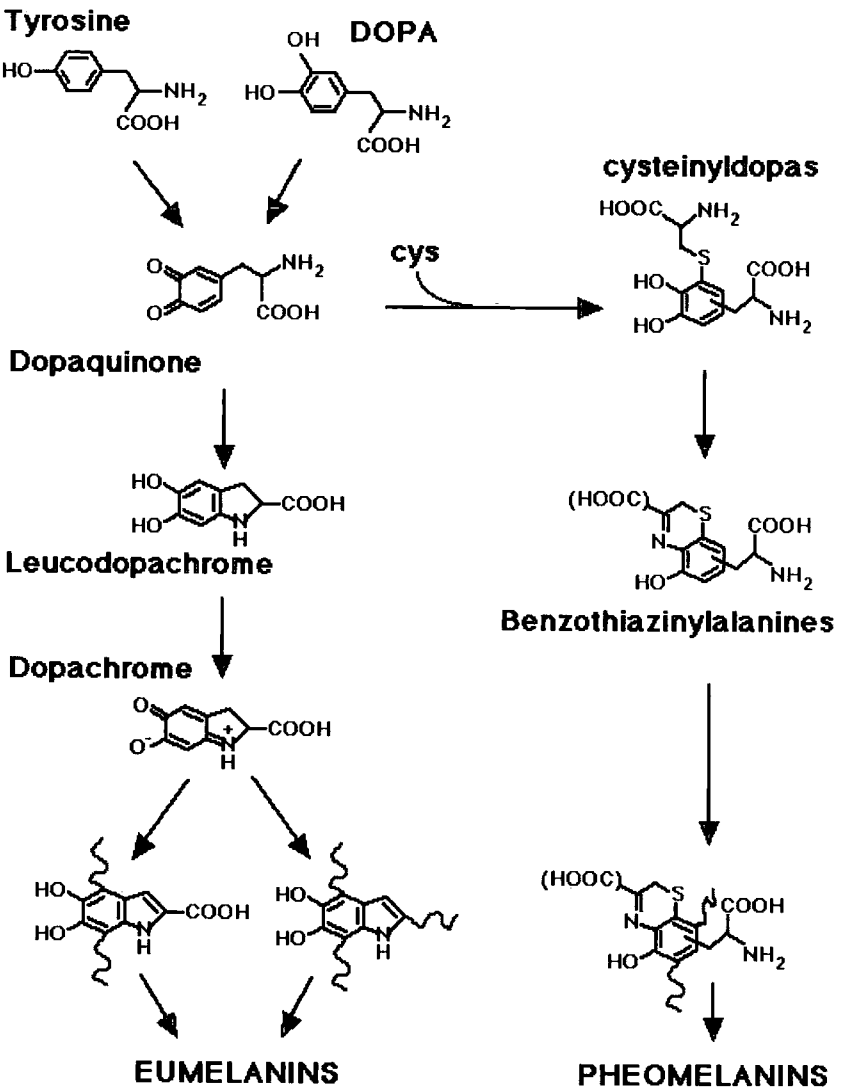
Although tyrosine has been widely reported to be a major determining factor for potato tuber tissues ability to produce blackspot bruise pigments, studies trying to correlate tyrosine levels and blackspot bruise susceptibility have been contradictory. Results from several groups indicate that levels of tyrosine are well correlated with bruising (Mapson *et al.*, 1963 ; Stark *et al.*, 1985 ; Sapers *et al.*, 1989 ; Corsini *et al.*, 1992 ; Dean *et al.*, 1992 ; Dean *et al.*, 1993 ; Stevens & Davelaar, 1997), while others suggest there is little or no correlation (Mulder, 1949 ; Schaller & Amberger, 1974). One of the more prominent papers in which tyrosine is reported to correlate well with bruising susceptibility is that of Sabba and Dean, 1994 in which a correlation of R=0.88 was reported between levels of free tyrosine and bruising susceptibility in certain varieties. This group suggested that higher levels of free tyrosine were present post mechanical impact due primarily to increased activity of endopeptidases and not due to increased traffic through the biosynthetic pathway for tyrosine. This perhaps suggests where some of the earlier results from groups had differed in so far as they were analysing key components of the biosynthetic pathway to tyrosine and could see no overall increase in synthesis leading to their conclusions that tyrosine and blackspot bruise intensity were not correlated. A more realistic proposition is that of Mondy and Munshi, 1993, who, although finding a positive correlation between bruising and free tyrosine within a cultivar, suggested that tyrosine levels were not the predominant factor in determining blackspot susceptibility in potatoes as a whole due to the fact that their cultivar with high blackspot bruise susceptibility had overall considerably lower levels of tyrosine than their cultivar with high blackspot bruise resistance. Studies by

Stark *et al.*, (1985) and Corsini *et al.*, (1992) noted that although levels of free tyrosine correlated well with blackspot bruise susceptibility there was an inverse relationship between the amount of protein-bound tyrosine with free tyrosine, suggesting that protein biosynthesis affected blackspot susceptibility by reducing the pool of free tyrosine avaible for use in by PPO. This was confirmed by Dean *et al.*, 1992 who used radio-labelled shikimic acid (an early component of the shikimate pathway), and observed that free tyrosine synthesis was 55% higher in the blackspot bruise susceptible cultivar Lehmi Russet, compared to the blackspot bruise resistant cultivar TXA763-5 – they could not identify any increase in labelled tyrosine incorporation into proteins when the two cultivars were compared, hence the pool of free tyrosine available to PPO was greater in the susceptible cultivar. In this context, level of spatial susceptibility to blackspot bruising also exists, with tubers being most susceptible at the stolon end of the tuber and least susceptible to discolouration at the bud end. Corsini *et al.*, 1992 showed that free tyrosine levels were 25% lower in the bud end compared with the stolon end and that bruise susceptibility was 29% lower at the bud end.

Total phenolic content (including caffeic and chlorogenic acids, which are both substrates for PPO and present in significant levels in the tuber) was correlated well with bruise susceptibility (R=0.75) however this was found to be mostly due to the tyrosine content and if tyrosine was removed the correlation broke down rapidly (R=0.13) strongly suggesting that tyrosine is the principal factor influencing susceptibility to blackspot bruise (Mapson *et al.*, 1963 ; Corsini *et al.*, 1992 ; Dean *et al.*, 1993). This finding was developed by Stevens *et al.*, 1998 who noted that as well as tyrosine, free cysteine can act as a well used co-substrate (leading to phaeomelanins) by studies upon the melanin end products (fig 1.1), though no correlation data between

Figure 1.1 : Synthesis of melanin. Steps from tyrosine to dopaquinone and from dopachrome to melanin are catalysed by polyphenol oxidase.

Image from http://omlc.ogi.edu/spectra/melanin/melaninsynth.gif



cysteine levels and blackspot bruise susceptibility were presented, the suggestion being that as cysteine was involved at a later stage in the pigment synthetic pathway a link between the two factors was unlikely. The same authors (Stevens & Davelaar, 1997) had noted that, although there was a strong correlation between potential blackening [the ability to produce dark pigments from a protein extract] and tyrosine (R=0.73) but not with chlorogenic or caffeic acids (R=0.03 / R=0.00 respectively), they could not find a similar correlation between tyrosine levels and potential blackspot [the actual ability to produce pigments as a response to mechanical stress] (R=0.06).

A threshold concentration of tyrosine of 4μmole per gram fresh weight has been proposed below which no bruise pigments are formed in response to mechanical impact (Corsini *et al.*, 1992). Above this level, up to between 175-200 μg tyrosine / g fresh tuber, blackspot potential appears to increase with increasing tyrosine levels. Above 175-200 μg tyrosine / g fresh tuber a saturation point is reached above which no further increase in blackspot bruise potential is observed with increasing tyrosine levels (Dean *et al.*, 1993)

1.3.3 Polyphenol Oxidase

Polyphenol oxidase [PPO] (also known as tyrosinase) is a copper-containing metalloprotein which catalyses the oxidation of monophenols to *o*-diphenols, and *o*-dihydroxyphenols to *o*-quinones. In the case of blackspot bruising, the pigment, melanin, arises through various polymerisation reactions of the *o*-quinone products with an assortment of cellular components including amino acids (Vaughn *et al.* 1988). Rather confusingly, even with recent changes in enzyme nomenclature, PPO retains

two EC numbers, one for each of the two distinct reactions catalysed, though both occur within the same enzyme:

1. EC1.14.18.1 - Hydroxylation of monophenols to *o*-diphenols [cresolase, tyrosinase, monophenol mono-oxygenase]

   Tyrosine + Oxygen → Dopaquinone (fig 1.1)

2. EC1.10.3.2 - Dehydrogenation of *o*-dihydroxyphenols to *o*-quinones [catecholase, diphenol oxygen oxidoreductase]

   Dopachrome + Oxygen → Melanin (fig 1.1)

Molecular studies on developing potato tubers have identified 5 separate PPO genes with high sequence similarity (Thygesen *et al.*, 1995). All of the genes encoded polypeptides of approximately 67kDa which included a 7kDa transit peptide – a common feature among PPO proteins from a variety of species (Cary *et al.*, 1992; Robinson & Dry, 1992; Shahar *et al.*, 1992; Hunt *et al.*, 1993; Newman *et al.*, 1993; Dry & Robinson, 1994). The five potato genes can be divided into three classes based upon sequence homology – classes I, II and III. The three classes have also been identified in tomato, and there is greater than 93% similarity between three potato clones and three corresponding genes in tomato (Thygesen *et al.*, 1995). In brief, the class I and class II PPO genes are expressed in non-photosynthetic tissues such as tubers, whereas the class III PPO genes are expressed in photosynthetic tissues. All PPO genes share extremely high sequence conservancy around a region of histidine-rich bases, which have been suggested to bind the copper atom into the protein structure (Dry & Robinson, 1994). PPO genes are nuclear encoded and the proteins are

usually located on the inner membranes of plastids (amyloplasts in the case of potato tubers). Although they are associated with the plastid membrane they are not, however, integral membrane proteins, indicating that they are not essential for membrane structural integrity though they may interact with the membrane components (Vaughn *et al.*, 1988). Within tomato chloroplasts the transport of PPO to the thylakoid lumen was shown to be a two stage process, the first stage involving the import of a precursor protein into the stroma followed by processing into a mature protein and translocation into the thylakoid lumen (Sommer *et al.*, 1994). Recent work by Partington *et al.*, (1999) using electron microscopy has suggested that PPO may also be found widely in the cytoplasm of non-damaged tubers, however the authors are cautious about this finding as this would imply a novel mechanism for preventing the reaction between PPO and tyrosine taking place under normal cellular conditions.

One of the principal roles proposed for PPO is as part of the plant defences against pathogens and phytophagous insects (Farkas & Kiraly, 1962 ; Kosuge, 1969 ; Felton *et al.*, 1989 ; Felton *et al.*, 1992 ; Kowalski *et al.*, 1992 ; Yu *et al.*, 1992). Insect attack leads to the plant synthesizing a range of 'proteinase inhibitor proteins' which interfere with the digestive system of the insect. It is widely held that an 18 amino acid polypeptide – systemin – acts as a mobile signal, initiating synthesis of the proteinase inhibitor proteins (Green & Ryan, 1972 ; Ryan, 1990 ; Pearce *et al.*, 1991 ; Orozco-Cardenas *et al.*, 1993 ; McGurl *et al.*, 1994 ; Narvaez-Vasquez *et al.*, 1994). In studies on tomato it was shown that PPO was strongly inducible by systemin, and also by methyl jasmonate – a key intermediate in the biochemical pathway to systemin production (the octadecanoid signalling pathway), further implicating the involvement of PPO in anti-insect defence processes (Constabel *et al.*, 1995). Exactly what role

PPO has in insect defence is, as yet, unknown, however the quinones previously mentioned as a reaction product have the ability to alkylate cysteine, histidine and lysine residues, thus reducing nutritional quality of proteins from the insects point of view (Pierpoint, 1983).

In potato tubers PPO has been shown to be most active in the epidermis and the first 1-2mm of the cortex which corresponds well with the tissues showing the highest bruising response. As the key enzyme involved in tissue browning, in a range of commercial fruit and vegetables, PPO has been an obvious target for inhibitor work with the goal of eliminating enzymatic discolouration. These studies are dealt with in section 1.3.4 as most also involve the intermediate dihydroxyphenylalanine. However, the development of antisense technology (in which gene sequences are reversed and reinserted into the genome with a promoter) allowed a potentially novel approach to be adopted. Bachem *et al.*, (1994) utilised antisense-PPO sequences to inhibit PPO expression in a number of transgenic potato lines. The group were able to reduce PPO levels to almost zero in the two varieties tested. It was found, however, that complete inhibition of PPO was necessary for browning to be completely eliminated since 95% reduction in PPO activity allowed a near normal phenotype of blackspot bruise to be observed. More recently Coetzer *et al.* (2001) undertook an anti-sense PPO study in which they found reduced levels of pigment production in 5 of 28 lines studied. These 5 lines all corresponded to lines of reduced PPO activity, though not, as previously suggested by Bachem, to lines of completely inhibited PPO activity. This technology is now currently under development at Monsanto and a number of test cultivars have now been developed eliminating all PPO activity and are nearing commercial release. The current low acceptance amongst the general public to genetically modified organisms

will, at present, cause some problems for the futher development and release of these transgenics in certain parts of the world. One particular consideration which will have to be addressed is what effect reduced PPO levels will have on other cellular processes – e.g. in pathogen resistance where PPO and the consequent deposition of melanin has been implied as a primary response to limiting pathogen spread – the production of a PPO deficient cultivar may thus be more susceptible to pathogen challenge.

Although tyrosine has been suggested as the key substrate for PPO in blackspot bruise, other potential monophenolic substrates are present in potato tubers – specifically caffeic acid and chlorogenic acid. Studies *in vitro* have noted that PPO oxidises the caffeic and chlorogenic acids more rapidly than tyrosine, however *in vivo* there is almost no correlation between monophenolics other than tyrosine and blackspot bruise potential (Raper & Wormall, 1923 ; Patil & Zucker, 1965 ; Craft, 1966 ; Stevens & Davelaar, 1997).

The use of molecular oxygen in the oxidation of the mono- and di- phenols is not universally accepted as some authors have demonstrated the superoxide free radical (section 1.4.2.2) is preferentially used *in vivo* (Wood & Schallreuter, 1991 ; Tobin & Thody, 1994 ; Valverde *et al.*, 1996). These findings have direct relevance to the work presented in this thesis and will be discussed more thoroughly in the results section.

1.3.4 Dihydroxyphenylalanine and quinone intermediates

The product of PPO enzymatic action upon tyrosine is dihydroxyphenylalanine (DOPA), a di-phenol which then undergoes a second reaction catalysed by PPO to yield dihydroxyphenylalanine quinone (DOPA quinone), an o-diquinone. From a food

quality point of view the presence of quinones results in a deterioration in potato flavour and nutritional quality (Friedman, 1995). Quinones are known to be able to bind to the protease enzymes in the digestive system of both insects and animals thus reducing the nutritional quality and safety of high-quinone containing compounds to foraging insects and human beings (Friedman, 1997).

As DOPA is a key intermediate between the two reactions catalysed by PPO when the initial mono-phenolic is tyrosine, it has formed the crux of research into anti-browning agents for use in the depletion of significant levels of rejection among many economically important fruits and vegetables. A range of naturally occurring compounds can act as browning inhibitors including carboxylic acids (Mayer & Harel, 1979 ; Passi & Nazzaro-Porro, 1981 ; Mayer, 1987). Sulphur containing compounds (especially those with thiol groups) are, however, widely held as the most potent inhibitors of PPO catalysed browning reactions, and have been demonstrated to be inhibitory in a wide range of systems including fruit and vegetable browning (Matheis, 1987b ; Dudley & Hotchkiss, 1989 ; Friedman & Molnar-Perl, 1990 ; Friedman *et al.*, 1991 ; Friedman *et* al., 1992 ; Sapers & Miller, 1992 ; Sanchez-Ferrer *et* al., 1993 ; Sapers, 1993 ; Golan-Goldhirsh *et al.*, 1994 ; Friedman & Bautista, 1995 ; Sapers & Miller, 1995). Thiols act in the system as reducing agents (antioxidants) which can reduce DOPA-quinone to form DOPA via DOPA-hydroquinone. Initially sodium sulphite was used as a reducing agent in food additives until several deaths were linked with the compound, which induces asthma attacks in susceptible individuals and has consequently been banned from use in most countries. Recently the use of cysteine has been investigated, which is already widely used as an antioxidant in foods (Friedman, 1996). Non-thiol containing antioxidants such as gallic acid have also been

demonstrated to be able to inhibit the PPO catalysed stage of the reaction from DOPA to melanin, these compounds reforming naturally occurring (PPO-inhibitory) carboxylic acids and derivatives (Kubo *et al.*, 2000) – gallic acids also being permitted for use as antioxidants in foods (Aruoma *et al.*, 1993).

1.3.5 Melanin

Melanin, a widespread plant and animal pigment, occurs in three principal forms; eumelanin, phaeomelanin and allomelanin. Eumelanin is the typical form of melanin in which the Raper-Mason reaction (section 1.3.2) takes place leading to a black-brown pigmented product (figure 1.1). Phaeomelanin occurs when nucleophilic addition of a thiol group occurs to dopa-quinone and thioether derivatives of dopa are produced, including cysteinyldopa which ultimately forms a red-brown sulphur-containing form of melanin. The third form of melanin, allomelanin, occurs when non-nitrogen containing diphenols are oxidised – these form a black melanin.

Eumelanin, is the predominant form (>95%) of melanin in potato tuber blackspot bruising, and will be discussed here. Key to the production of eumelanin is the initial hydroxylation of L-tyrosine to DOPA, followed by oxidation of DOPA to DOPA-quinones, catalysed by PPO as discussed in section 1.3.3. The DOPA-quinones then either undergo decarboxylation to 5,6-dihydroxyindole (DHI) or tautomerization to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) (Prota, 1992 ; Prota, 1997). Eumelanin is then formed by the oxidative condensation of DHI and DHICA into a polymer (Jimbow *et al*,. 1984 ; Sealy, 1985 ; Nordlund *et al.*, 1989 ; Tsukamoto *et al.*, 1992), either by the use of PPO or peroxidase (POD) (D'Ischia *et al.*, 1991 ; Shibata *et al.*, 1993 ; Palumbo & Jackson, 1995 ; Palumbo *et al.*, 1997). Eumelanins (indeed melanins in

general) possess a diverse range of properties. Commercially the most important is the role of the compound as a photoprotectant as used in sunscreens. The molecule has a photoprotectant property because it permanently possesses free electrons which originate from the incomplete oxidation of the DHI and DHICA compounds. As a consequence of incomplete reactions the DHI and DHICA appear to exist in equilibrium with the partially oxidised (semi-quinone) forms, known as the 'semiquinone radical' form due to the additional free electrons (Reszka *et al.*, 1998). Because of this equilibrium the molecule has excellent electron absorbing properties, and can act as an intermediate in redox-electron reactions both absorbing and donating electrons. Light, changes in pH and interactions with potent redox compounds have all been shown to alter the semiquinone radical equilibrium and it is in this way that melanin is believed to act as a photoprotectant by scavenging free radicals, known to be produced by the action of ultra-violet light on proteins and several other molecules (Chauffe *et al.*, 1975 ; Felix *et al.*, 1978 ; Crippa *et al.*, 1979 ; Sealy, 1984 ; Oniki & Takahama, 1992 ; Bustamante *et al.*, 1993 ; Reszka & Jimbow, 1993 ; Zughaier *et al.*, 1999). In plants the radical scavenging role of melanin has been linked with a different system. Upon attack by a pathogen (fungal or bacterial), the plant cells release large quantities of oxygen-based free radicals (reactive oxygen species (ROS) into the extracellular spaces as part of a battery of responses aimed at restricting and inhibiting the spread of the pathogen (section 1.4). Work by Jacobson and Hong, (1997) demonstrated that *Cryptococcus neoformans*, a black-pigmented fungal pathogen, utilises melanin to 'buffer-out' the free radicals being produced by the plant, similar animal pathogens are also known. This suggests that the melanin may play a role in the protection of plant cells against attack by ROS generated in response to pathogen challenge.

Contrary to its role as a radical scavenger melanin also has the ability to generate free radical species, including superoxide (the roles of which are discussed in section 1.4) (Sarna *et al.*, 1980 ; Korytowski *et al.*, 1987 ; Hill, 1992 ; Qu *et al.*, 2000). Intriguingly superoxide has been implicated as a preferred PPO co-substrate instead of molecular oxygen, in melanogenesis in mouse melanoma cells (and also in work presented in this thesis), leading the authors to suggest that the biosynthesis of melanin could act as a free radical trapping mechanism (Valverde *et al.*, 1996). This suggests a potentially complex cycling of free radicals, with the melanin both scavenging and producing radicals, as well as utilising them in the formation of more melanin.

Eumelanin has long been held to be the end-product of mechanical impact upon potato tubers as described by the biosynthetic pathway in sections 1.3.1 to 1.3.3. This, however, has been recently challenged by Stevens *et al.*, 1998, who identified benzothiazine derivatives of cysteinyldopa (figure 1.1), indicating the incorporation of cysteine (and potentially glutathione) into melanin suggesting that at least part of the melanin component of blackspot bruise may be phaeomelanin – the melanin produced by interaction of DOPA with thiol-containing compounds. They further suggest that the only effective way of controlling blackspot is thus by preventing the initial quinone being formed, e.g. by anti-PPO – a conclusion not entirely borne out by current studies on quinone trapping to prevent melanin formation.

1.4 Biologically Important Free Radicals

1.4.1 Overview

Plants and animals have developed a range of defence mechanisms against invading pathogens. One of the most rapid defences is the production of excess quantities of highly reactive products derived from molecular oxygen, known as the oxidative burst. These products include free radicals of oxygen ($O_2^{-}\cdot$, $HO\cdot$) and hydrogen peroxide and are classed together under the title 'active oxygen species' (AOS) and are shown in table 1.7

Free radicals, being highly reactive are extremely damaging to many biological systems and are therefore very short lived. Due to the damaging effects of the AOS on the cells and tissues of the generating species, an array of neutralising systems exist within plants and animals to permit their safe removal. Foremost among the free radical scavenging enzymes is superoxide dismutase (SOD) which is found in at least 3 forms using various metal ions as cofactors. SOD disproportionates superoxide radicals into hydrogen peroxide and oxygen (reaction 1).

Reaction 1 : $2\ O_2^{-}\cdot + 2\ H^{+} \rightarrow H_2O_2 + O_2$

Catalase primarily detoxifies hydrogen peroxide within the cell by converting it into water and oxygen (reaction 2).

Reaction 2 : $2\ H_2O_2 \rightarrow H_2O + O_2$

Table 1.7 : **Active oxygen species.** Structures of active oxygen species

Active Oxygen Species	Structure
SUPEROXIDE RADICAL	$O_2^{-\cdot}$
HYDROXYL RADICAL	$OH^{\cdot}$
HYDROPEROXYL RADICAL	$H_2O^{-\cdot}$
HYDROGEN PEROXIDE	H_2O_2

Finally peroxidases transfer the electrons away from radicals onto various accepting compounds, which predominantly are metal-ion electron acceptors (Reaction 3).

Reaction 3 : $_{red}\text{compound} + H_2O_2 \rightarrow {}_{oxid}\text{compound} + H_2O$

These three enzyme families permit almost all metabolically generated AOS to be safely scavenged and thus reduce their deleterious effects on cells. In the case of the oxidative burst, the scavenging systems, are, however, transiently overwhelmed and are unable to remove the AOS quickly enough to prevent their reaction with important molecules.

1.4.2 Generation

1.4.2.1 Biological generation of free radicals

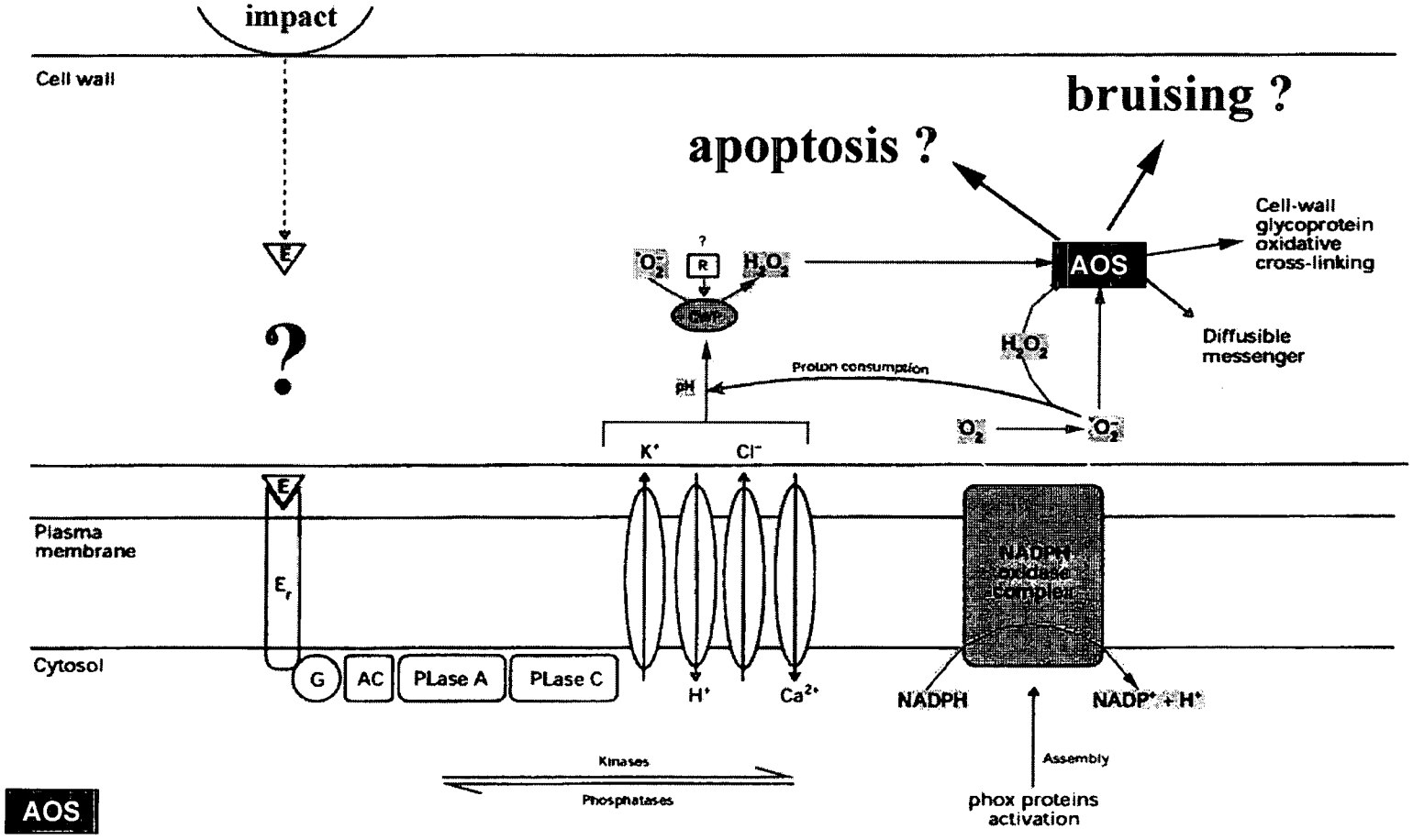
The interaction of AOS and living cells is potentially a very damaging event in all organisms. The oxidative burst was first identified in mammalian neutrophils, white blood cells associated with combating disease, and shortly after was described in plant cells, responding to pathogen challenge (Doke, 1983). AOS have been demonstrated to have a negative effect in a wide range of human diseases including arthritis, heart disease, stroke, AIDS, emphysema, ulcers, hypertension, preeclampsia, multiple sclerosis, Alzheimers disease, Parkinsons disease, muscular dystrophy, alcoholism, cancer and others (McCord, 1974 ; Cohen, 1984 ; Lyras *et al.*, 1984 ; Dianzani, 1985 ; Vaille *et al.*, 1990 ; Omar & McCord, 1991 ; Davies *et al.*, 1992 ; Toshniwal & Zarling, 1992 ; Flores *et al.*, 1993 ; Wallaert *et al.*, 1993 ; Asami *et al.*, 1997 ; Ragusa *et al.*, 1997 ; Baker *et al.*, 1998 ; Aguirre *et al.*, 1999 ; Hubel, 1999 ; Kerr *et al.*, 1999).

Two separate mechanisms for the generation of active oxygen species (AOS) have been proposed in plant systems (fig 1.2).

The first system is one which has strong similarity to the mammalian neutrophil oxidative burst generation system (Babior *et al.*, 1973, Babior, 1978). In this system the presence of a transmembrane NADPH-oxidase was demonstrated with free radical generation depending on intracellular signalling cascades involving GTP-binding proteins, ion channels, protein kinases and phosphatases, phospholipase A and C as well as cAMP. The core of the NADPH oxidase in mammals is a heterodimeric flavocytochrome b_{558} which has NADPH-binding properties. Cyt b_{558} is associated with both a glycosylated transmembrane protein $gp91^{phox}$ and a non-glycosylated $p22^{phox}$ subunit. b558 contains an entire electron transfer chain and the other protein components apparently play only structural and regulatory roles. The oxidase is activated by the phosphorylation of two other proteins ($p40^{phox}$ and $p67^{phox}$) which translocate to the membrane together with $p47^{phox}$ and $p21^{rac}$, the latter being a GTP-binding protein (Reviews : Segal & Abo, 1993 ; Jones, 1994 ; Wientjes & Segal, 1995 ; Shatwell & Segal, 1996). The complete translocated conjugate is the active NADPH oxidase, which initiates generation of superoxide by reaction 4. The strongest evidence for the existence of this compound in plants is the presence of the gene *rboh A* which was isolated from rice and has very strong sequence similarity to $gp91^{phox}$ (Groom *et al.*, 1996).

Reaction 4 $\quad NADPH + 2O_2 \rightarrow NADP^+ + 2O_2^{\cdot-} + H^+$

Figure 1.2 : Proposed mechanisms for generation of active oxygen species in plants. Abbreviations used AC – Adenylate Cyclase ; AOS – Active Oxygen Species ; CWP – Cell Wall Peroxidase ; E – Elicitor ; G – G Protein ; PLase - Phospholipase. Adapted from Wojtaszek, 1997.



Work using the NADPH-oxidase inhibitor diphenylene iodonium (DPI) has lead to further evidence of a role for the NADPH-oxidase generating system for AOS, in which the oxidative burst as a response to challenge by bacterial pathogens or elicitors has been eliminated in several plant species (Levine *et al.*, 1994 ; Auh & Murphy, 1995 ; Desikan *et al.*, 1996 ; Dwyer *et al.*, 1996 ; Murphy & Auh, 1996 ; Jabs *et al.*, 1997 ; Mithofer *et al.*, 1997). It should, however, be borne in mind that DPI possesses a degree of inhibitory activity over the proposed alternative mode of ROS generation in plants thus direct conclusions from the use of DPI are to be avoided.

The alternative method for ROS generation is one which has no parallels in animal systems. In this system greater emphasis is placed upon ion channel movements. Depolarization is a well characterized response immediately prior to the oxidative burst and typically Ca^{2+} and H^{+} influx from the extracellular matrix (ECM) into the cytosol occurs with a corresponding K^{+} and Cl^{-} efflux into the ECM. These ion fluxes lead to a transient alkalinisation of the apoplastic fluid, which is demonstrable in cell suspension cultures treated with elicitors (Abdullah, 1998). This change in pH leads to the activation of a proposed pH-dependent cell wall peroxidase. The resultant conformational change in the active site of the peroxidase leads to peroxidase compound III (Fe^{2+}-O-O) being produced and subsequently hydroxyl radicals ($OH^{\cdot}$) which can be reduced initially by oxidation of cysteine residues close to the haem group in the enzyme (Lindner *et al.*, 1988 ; Peng & Kuc, 1992 ; Bolwell *et al.*, 1995 ; Bolwell, 1996 ; Bestwick *et al.*, 1997). The main evidence for this mode of AOS generation comes from Bolwell *et al.*, 1995 who used ionophores and strong buffers to dissipate the pH change. They found that when the pH change was dissipated the

oxidative burst was substantially reduced, and no NADPH was oxidised (as was observed in the NADPH oxidase driven mechanism).

Given the evidence a combination of mechanisms incorporating both the NADPH-oxidase and the pH-dependent cell wall peroxidase mechanisms would seem the most likely mode of generation of AOS in plants, and it is in this combined approach that a number of researchers are currently working.

1.4.2.1.1 Characteristics of AOS generation

When a microbial elicitor is used to initiate an oxidative burst in plants a characteristic biphasic production (two distinct peaks of superoxide production separated by a period of lower production) of superoxide radicals is observed (Levine *et al.*, 1994 ; Baker & Orlandi, 1995). The first phase, which is typically only 50-75% the magnitude of the second, occurs 1-2 hours post elicitation and has been ascribed to a non-specific response to stress. The second phase which occurs 4-6 hours post-elicitation has been proposed to be a specific interaction between resistance conferring (R) genes and avirulence genes (avr) carried by the plant and the pathogen respectively, the interaction of these factors leads to either a resistance or disease phenotype 'the gene for gene hypothesis', as first described by Flor, 1947. It is thought to be the R/avr gene interaction perhaps via a transmembrane receptor which leads to a signalling cascade ultimately activating the G-proteins of NADPH-Oxidase. This aspect is related to results reported later in this thesis and will be discussed in more detail in these sections.

1.4.2.2 Chemical generation of free radicals

The oxygen molecule (O_2) is already a radical, this definition being applied to any compound which has a single unpaired electron in its atomic structure, oxygen having two unpaired electrons each residing in its own orbital. Thermodynamically the most stable form for oxygen is in water where all orbitals contain spin-paired electrons. The inability of oxygen to readily convert to water due to insufficiency of time during collisions between molecules is what saves us and most other organic life from spontaneously reacting in an explosive manner in an atmosphere of high oxidising potential. The ability of oxygen however to obtain electrons by auto-oxidation is however, possible, in this way it can only accrue electrons one at a time. The accumulation of single electrons into molecular oxygen leads to the production of free radicals (see review by McCord, 2000). One electron addition to oxygen leads to superoxide radical production (Reaction 5).

Reaction 5 $e^- + O_2 \rightarrow O_2^{-\cdot}$

A further electron addition leads to hydrogen peroxide (reaction 6) which is electron-stable and thus is not a radical. Hydrogen peroxide has a high ability to recruit a further two electrons to reach a higher stability and is thus a potent oxidising agent (reactions 7,8).

Reaction 6 $O_2^{-\cdot} + e^- + 2H^+ \rightarrow H_2O_2$

Reaction 7 $H_2O_2 + e^- + H^+ \rightarrow HO^{\cdot}$

Reaction 8 $HO^{\cdot} + e^- + H^+ \rightarrow H_2O$

Iron and copper ions in trace quantities are able to transfer a third electron to hydrogen peroxide which cleaves the oxygen-oxygen bond and yields the hydroxyl free radical (Reaction 9). The hydroxyl radical is extremely potent and can spontaneously and rapidly oxidise almost any organic molecule.

Reaction 9 $$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + HO^{\cdot} + OH^{-}$$

(see review by Wojtaszek, 1997)

1.4.3 Actions of Free Radicals

The array of reactions in which AOS have been implicated is vast and constantly expanding. This diversity of reactions can largely be explained by the substantive evidence implicating AOS in the activation and regulation of a number of key signalling molecule types (Chen *et al.*, 1993 ; Cimino *et al.*, 1997 ; Jabs *et al.*, 1997 ; Lamb & Dixon, 1997 ; Lander, 1997 ; Schulze-Osthoff *et al.*, 1997). AOS have been shown to activate a number of transcription factors via signalling cascades, including AP-1, NF-κB and p53 (Schulze-Osthoff *et al.*, 1995 ; Cimino *et al.*, 1997). They have also been shown to activate a range of kinases, including protein kinase C, MAP kinases, tyrosine receptor kinases and Src kinases (Klann *et al.*, 1998 ; Abe *et al.*, 2000 ; Herrlich & Bohmer, 2000). It also proposed that AOS may interact with the regulation of some stress induced kinases by adjusting the balance of the redox state of thioredoxin (Sen & Packer, 1996 ; Saito *et al.*, 1998 ; Adler *et al.*, 1999). G-protein activity is also thought to be dependent upon redox state and thus AOS may also have a regulatory role here (Lander, 1997). A further contributory factor is the large diversity in the chemical reactions of AOS with other molecules (proteins etc.) and the consequent functional changes conferred by structural change.

Due to the wide range of signalling molecules whose activity may be modulated by AOS it is not surprising that an equally large range of responses have been shown to be controlled, in some degree, by AOS. Within plants, the primary role of AOS is believed to be in defence against pathogens. Release of AOS has been shown to be the first response to microbial elicitation, further studies have demonstrated a pathogen defence role for AOS as

- an inducer of systemic acquired resistance
- an inducer of the hypersensitive response
- an initiator of oxidative cell-wall cross-linking reducing its susceptibility to enzymic digestion
- an active toxin against micro-organisms
- an initiator of programmed cell death (apoptosis)

(Bradley *et al.*, 1992 ; Peng & Kuc, 1992 ; Chen *et al.*, 1993 ; Brisson *et al.*, 1994 ; Levine *et al.*, 1994 ; Tenhaken *et al.*, 1995 ; Wojtaszek *et al.*, 1995).

AOS have been widely implicated in causing lipid membrane disruption. Lipases and lipoxygenase interact to cause lipid peroxidation by catalysing the peroxidation of linoleic and linolenic acids (these polyunsaturated fatty acids being particularly abundant in potato tubers). In blackspot bruising, membrane integrity is of importance in controlling the degree of decompartmentalisation (i.e. the degree of mixing of substrate and enzyme) (Laerke *et al.*, 2000). Relative differences between potato varieties in susceptiblility to lipid peroxidation can thus be inferred to be of high importance in defining varietal susceptibility to blackspot bruise.

One of the most interesting of the proposed roles for AOS is as an inducer of programmed cell death (PCD) or apoptosis in which cells undergo a controlled series of changes leading ultimately to death – in this way inhibiting spread of, for example, a pathogen. Apoptosis has been demonstrated in both plants and animals and has been characterised as a three phase process : induction , effection and degradation. It has been shown to be present both as a mechanism for the removal of unwanted or defective cells in animals (Ellis *et al.*, 1991) and in plants (for example in gamete and other cell development) and as a mode of control of pathogen spread (Jones & Dangl, 1996 ; Beers, 1997 ; Fukuda, 1997 ; Morel & Dangl, 1997). Apoptosis is also the process by which entire plant organs are removed during senescence (Hadfield & Bennett, 1997). Evidence for the involvement of AOS in apoptosis comes from a range of experimental observations. Exogenous application of $HO^{\cdot}$, $O_2^{\cdot-}$ and H_2O_2 have all been shown to induce apoptosis (Levine *et al.*, 1994 ; Jacobson & Raff, 1995 ; Jabs *et al.*, 1996 ; Zettl *et al.*, 1997). Ultra-violet light, a known promoter of radical production (section 1.3.5), has also been shown to induce apoptosis (Gorman *et al.*, 1997). Radical scavengers can inhibit the activation of apoptosis, e.g. superoxide dismutase (Bowler *et al.*, 1991 ; Greenlund *et al.*, 1995 ; Jabs *et al.*, 1996 ; Van Camp *et al.*, 1996 ; Gorman *et al.*, 1997 ; Prehn *et al.*, 1997), catalase (Sandstrom & Buttke, 1993 ; Behl *et al.*, 1994 ; Levine *et al.*, 1994 ; Busciglio & Yankner, 1995 ; Jacobson & Raff, 1995 ; Verhaegen *et al.*, 1995 ; Chandra *et al.*, 1996 ; Johnson *et al.*, 1996 ; Mills *et al.*, 1996 ; Gorman *et al.*, 1997 ; Lin *et al.*, 1997) and the NADPH-oxidase inhibitor diphenylene iodonium (Sandstrom & Buttke, 1993 ; Levine *et al.*, 1994 ; Chandra *et al.*, 1996 ; Jabs *et al.*, 1996). Given this evidence a role for AOS in apoptosis is largely proven (Jabs, 1999).

It can thus be seen that potato blackspot bruising and the generation of active oxygen species have a wide range of influencing factors – this work addresses both areas and implies a direct interaction between both aspects – a previously undiscovered connection.

AIMS & OBJECTIVES

2 Aims and objectives

Blackspot bruise is the result of impact damage, the level of which is genetically determined (i.e. cultivar dependent). Oxidative burst is a mechanical stress response which is also genetically determined. Therefore this thesis set out to test the hypothesis that synthesis of blackspot bruise pigments is quantitatively determined by the level of generation of active oxygen species. The hypothesis was tested by addressing the following aim and objectives.

The aim of the project was to try to establish a relationship between biochemical factors and levels of susceptibility to blackspot bruising.

The project had several specific objectives:

1. To demonstrate the presence of modified proteins resulting from exposure to active oxygen species.

2. To assess the production of active oxygen species as a consequence of mechanical impact

3. To establish any correlation between blackspot bruise susceptibility and factors associated with oxidative stress

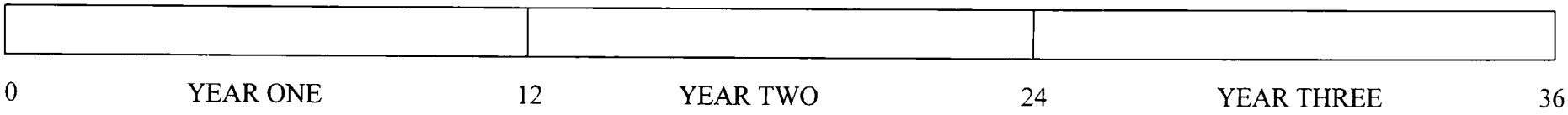
4. To investigate the role of polyphenol oxidase and tyrosine in the production of blackspot bruise pigments and to relate these to active oxygen species production.

5. To investigate the influence of various metal ions on the development of blackspot bruise pigment formation.

2.1 Progress

Progress through the project is shown in figure 2.1. OxyBlot analysis took a disproportionately large period of time to complete as the method proved problematical in its application to this system, discussed in section 4.2.5.

Figure 2.1 : Progress through the project



Experimental areas studied

Bruise index assays	OxyBlot analysis	Spectro-method for carbonyls	XTT-based assays	Inhibitor studies	PPO activity assays	Metal Ions	Multiple Bruising

METHODS & MATERIALS

A list of consumables and chemicals can be found in section 3.10

3 Methods

3.1 Mechanical impact damage to potato tubers

A fundamental technique was the introduction of standardised mechanical damage to potato tuber material. A standardised protocol was established to ensure reproducibility of bruised material and to ensure all impacted material was subjected to the same energy of impact.

Prior to mechanical impact potato tubers were carefully washed in cool water and carefully dried to remove excess soil from the epidermis, whilst minimising mechanical damage by careful handling of tubers. Tubers were then incubated at 4°C for 48 hours to allow cold temperature acclimatisation. It has been well established that cold acclimatised tubers display enhanced blackspot bruise formation. Tubers were then either bruised with a single impact (section 3.1.1) or with multiple impacts (section 3.1.2). The site of mechanical impact was carefully marked on the tuber epidermis with a permanent marker before transferring the tubers to 27°C for bruise development; in the majority of experiments this was for a maximum of 48 hours however in certain experiments the incubation time was reduced to allow only partial development of the blackspot bruise to be observed or for time course experiments.

3.1.1 Single impact

Tubers were prepared as in section 3.1 before being subjected to a mechanical impact involving a metal bolt of 240g falling through a height of 300mm imparting an energy of 0.72J – the 'falling bolt' method. The energy imparted was calculated according to equation 3.1.

3.1 Energy = Mass (Kg) x Gravity (N) x Height (m)

0.24Kg x 10N x 0.3m = 0.72J

In the case of a single impact the tuber was positioned so that the point of impact of the falling bolt would be as close as possible, without actually being directly on, the stolon entry point of the tuber. Occasionally depending upon the nature of the experiment the point of impact was varied to allow blackspot bruise susceptibility comparisons to be made.

3.1.2 Multiple impacts

Where multiple impact sites were required on the same tuber, the same 'falling bolt' method was adopted in which an energy of 0.72J was imparted as before. However attention was paid to the fact that a secondary bruise frequently forms at the point *opposite* the point of impact where the tuber was resting on a hard surface. Thus multiple impacts were imparted at parts in the same plane and on the same side of the tuber to ensure that no primary bruises could be affected by secondary '*opposite*' bruises.

3.2 Blackspot bruise assessment

In order to obtain a valid assessment of blackspot bruise susceptibility within a certain variety a standardised bruise assessment test was carried out. 25 tubers per variety

were mechanically impacted using the single-impact technique (section 3.1.1). After complete bruise development (48 hours) each tuber was transected through the site of mechanical impact and a measure of the width and depth of pigmented tissue was taken along with a visual assessment of bruise pigment depth of colour compared with the surrounding tissue (fig 3.1), using the following scale:

0 – No visible pigmented tissue at site of mechanical impact

1 – Low level of pigmented tissue (typically pink, red or red-brown)

2 – Intermediate level of pigmented tissue (typically brown or brown-black)

3 – High level of pigmented tissue (typically blue-black or black)

The mean for bruise depth, width and pigment assessment was calculated and this was used for comparing blackspot bruise susceptibility between different varieties, or between different storage ages of tubers from the same variety.

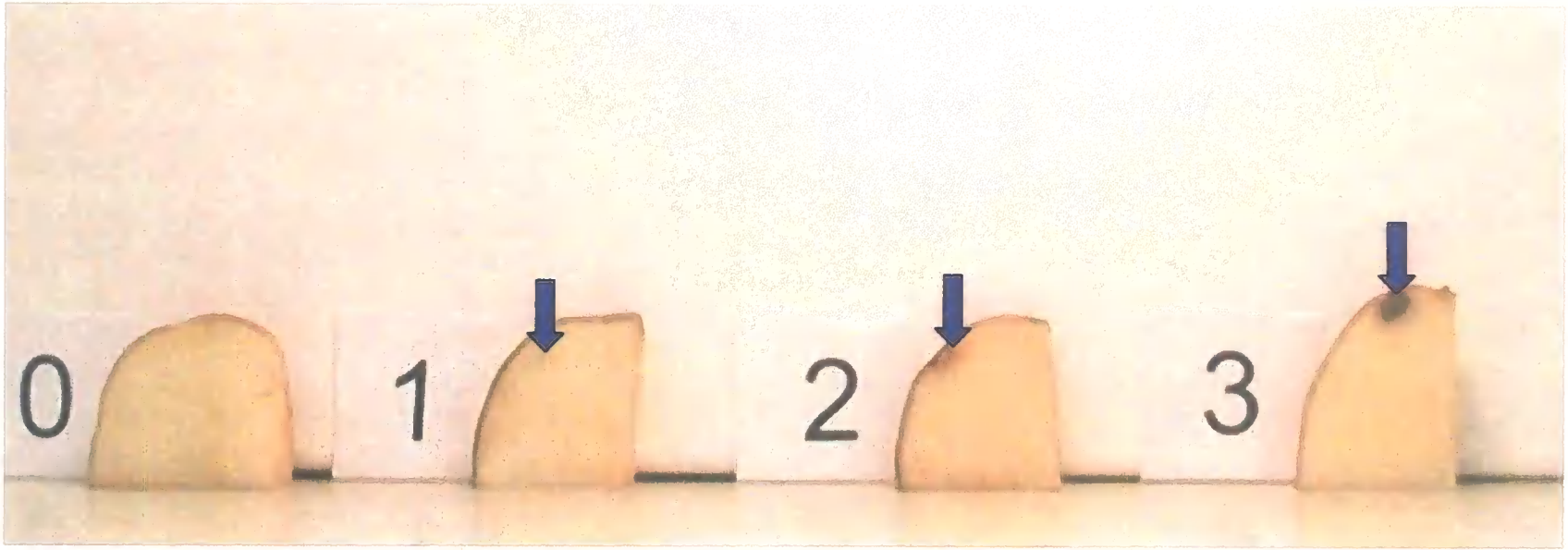
Bruise index was calculated by using equation 1. This assumes a cylindrical shaped bruise and by dividing by 235.6 this compares the bruise index to a bruise with diameter 10mm, depth 10mm and bruise intensity 3 – theoretically a highly intense bruise and one not observed in practice.

Equation 1

$$\frac{\pi \times \tfrac{1}{2}(\text{bruise width})^2 \times \text{bruise depth} \times \text{bruise intensity}}{235.6}$$

This method of calculating bruise index has several advantages over previously used methods – it factors in size of the bruise as well as bruise intensity, and further it allows for easy comparison of bruise indices on a scale of 0-10.

Figure 3.1 : Degree of discolouration for visual assessment of bruise index. Tubers of Russet Burbank showing variations in degree of discolouration from 0 (no discolouration) to 3 (deep discolouration). The position of the bruise is marked by an arrow in each case.



3.3 Preparation of protein extracts

A range of protein extracts were required for many of the experiments undertaken. Protein extraction was standardised dependent upon the requirements of each individual experiment.

Total protein extracts were prepared from potato tuber tissue as follows - 125mm^3 of subdermal tissue was excised with a clean razor blade and weighed. Sample buffer (125mM tris-HCl pH 6.8, 0.01% (v/v) β-mercaptoethanol) was added to the excised tissue at a ratio of 1:4 and the tissue homogenised on ice using an eppendorf tube plastic homogeniser. The resulting extract was centrifuged (benchtop eppendorf centrifuge, 15000g, room temperature, 5 mins) and the supernatant decanted. After retaining samples (300µl) for protein quantification (section 3.3.1), the remainder of the supernatant was stored at -80°C or, alternatively, had 66% (v/v) sample buffer (6% (w/v) sodium dodecyl sulphate (SDS), 10% (v/v) glycerol and 0.05% (w/v) bromophenol blue) added if required for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The protein preparation was either used directly or stored at -80°C until required.

3.3.1 Bradford assay for protein quantification

Before running gel electrophoretic analysis it was necessary to quantify protein levels in the protein extracts. To do this the methods of Bradford (1976) and Spector (1978) were employed. Samples were prepared as described in section 3.3 and the separated supernatant without added SDS, glycerol or bromophenol blue was used for the assay

(both SDS and bromophenol blue interfere with this assay which is dependent upon a colour shift of Coomassie Brilliant Blue G-250 upon protein binding).

A range of bovine serum albumin (BSA) concentrations was prepared (0.1μg-10μg) and a standard curve prepared. 100μl of sample or BSA standard was added to 900μl of Bradford reagent (BioRad, Poole, UK) in a micro-cuvette and incubated at room temperature for 10 mins. The absorbance at 595nm was then read using a Helios spectrophotometer (UK) against a 90% (v/v) Bradford reagent blank. Protein concentrations of samples were calculated from the BSA standard curve using GraphPad Prism (Prism, US).

3.3.2 Protein extraction for assay of oxidative modification

125mm^3 of tuber subdermal tissue protein extracts (section 3.3) which were to be derivatised using dinitrophenylhydrazine (DNPH) were extracted into derivatisation buffer (50mM HEPES pH7.4 ; 100mM KCl ; 10mM $MgCl_2$; 25μM $FeCl_3$), and not sample buffer as above.

3.4 SDS-Polyacrylamide Gel Electrophoresis (PAGE)

SDS-PAGE was employed widely, both for direct comparisons of polypeptide components in different samples and also for Western blotting (section 3.5.2) for the detection of oxidatively modified proteins.

SDS-PAGE was performed according to the method of Laemmli (1970) using a BioRad Mini-Protean II kit (BioRad, Poole, UK). 0.75mm thick gels were cast, using a 12% (w/v) acrylamide / bis-acrylamide (37.5:1) resolving (375mM tris-HCl pH8.8 ;

0.1% (w/v) SDS) and 4% (w/v) acrylamide / bis-acrylamide (37.5:1) stacking gel (125mM tris-HCl pH6.8 ; 0.1% (w/v) SDS). Samples prepared as in section 3.3 were denatured at 95°C on a heating block for 5 minutes and were electrophoresed at 70v until the dye front reached the stacking / resolving gel interface and then at 170v until the dye front reached the base of the gel. Gels were run in buffer (0.124M tris-base pH8.3 ; 0.96M glycine ; 17.36mM SDS). Where appropriate gels were stained in Coomassie Brilliant Blue R-250 solution (0.1% (w/v) Coomassie Brilliant Blue R-250 ; 40% (v/v) methanol ; 10% (w/v) acetic acid), followed by destaining in 40% (v/v) methanol, 10% (v/v) acetic acid. Gel images were recorded by scanning using a Hewlett Packard 11cx scanner and saved on computer.

3.5 Production of active oxygen species

3.5.1 Chemical generation of superoxide radicals

Superoxide radicals were generated chemically by the interaction of ferric chloride and ascorbate (Levine, 1980). 1.28M ascorbate (in 100mM HEPES pH7.0) was added to 50mM HEPES pH7.4 (containing 25μM $FeCl_3$) at a ratio of 20μl ascorbate : 1ml ferric chloride solution. Superoxide is generated for approximately 40 mins, however in practice generation periods of less than 25 mins were used throughout, using 1mM EDTA to terminate the reaction at the desired time (Levine, 1980).

3.5.2 Derivatisation of proteins with DNPH

Potato samples were analysed for the level of protein oxidative modification. Proteins were analysed for levels of secondary carbonyl groups, the characteristic structural alteration caused by exposure to superoxide radicals. Proteins in extracts (section 3.3) were first derivatised with dinitrophenylhydrazine (DNPH) which reacts with

secondary carbonyl groups to yield hydrazone derivatives with the proteins. Proteins were extracted (section 3.3.2) and were derivatised as follows : 5µl 12% (w/v) SDS was added to 5µl of protein extract. To this, 10µl 20mM DNPH in 10% (v/v) trifluoroacetic acid (TFA) was added (or 10µl 10% (v/v) TFA in the control). After exactly 20 minutes at 16°C the reaction mixture was neutralized by the addition of 7.5µl neutralization solution (2M Tris, 30% glycerol, 0.74M 2-β-mercaptoethanol). The time for derivatization was crucial, as less than 15 mins leads to incomplete derivatization, whereas greater than 25 mins leads to side reactions leading to erroneous estimates (Levine *et al* 1994).

3.5.3 Western Blotting

After derivatization of proteins with DNPH (section 3.5.2), SDS-PAGE was carried out (section 3.4) to fractionate the polypeptides and these were then transferred to a nitrocellulose membrane by Western blotting. Derivatised proteins were detected using anti-DNP hydrazone antibodies. Western blotting was carried out using a BioRad Mini-Protean II Western Blotting Kit (BioRad, Poole, UK).

Prior to blotting, the nitrocellulose membrane was pre-wetted in Western transfer buffer (0.12M Tris-base ; 0.1M glycine ; 20% (v/v) methanol pH8.3) for 5 minutes. The PAGE gel to be blotted was pre-wetted in Western transfer buffer for 15 minutes. Assembly of the Western blotting apparatus was carried out according to the BioRad instruction manual. Briefly this involved placing the pre-wetted gel and nitrocellulose membrane in contact with each other, removing any air bubbles between the gel and membrane and placing these between six pieces of Whatmann 3mm filter paper cut to the exact size of the membrane and gel (to prevent 'short-circuiting' of transfer buffer).

The gel was electro-blotted for 60 mins at 100 v. After blotting the Western blot was stained with Ponceau R stain for 2 mins to confirm protein transfer had taken place. Prior to antibody probing the membrane was extensively washed with deionised water to remove all traces of the Ponceau R stain.

3.5.4 Antibody probing and detection

Oxidatively modified, DNPH-derivatised proteins were detected on Western blots by using anti-DNP hydrazone antibodies, followed by detection using a luminol-based chemiluminescence kit.

Western blots on nitrocellulose membranes were reacted with specific anti-DNP hydrazone antibodies as follows. The blot was initially treated using 'blocking buffer' (1% (w/v) BSA in PBS-Tween (0.14M NaCl ; 2.7mM KCl ; 4.3mM $Na_2HPO_4.12H_2O$; 1.5mM KH_2PO_4 ; 0.05% (v/v) Tween 20)) for 1 hour at room temperature (1ml/30cm^2). The blots were then probed with anti-DNP hydrazone antibody (rabbit) for 1 hour at room temperature (1:300 in blocking buffer). Blots were then washed for 30 minutes in excess PBS-Tween buffer with 5 changes at room temperature. Blots were the incubated with peroxidase linked anti-rabbit secondary antibodies (goat) for 1 hour at room temperature (1:600 in blocking buffer). A final 30 minute series of washes was carried out in PBS-Tween (5 changes at room temperature).

Chemiluminescent detection of bound peroxidase-antibodies was carried out using a Boehringer Mannheim Peroxidase Chemiluminescence Kit (POD). The luminol substrate based kit was prepared for use as described the instruction manual, 30

minutes prior to developing the membrane and kept in the dark at room temperature. The washed blot and chemiluminescence kit were then transferred to dark-room facilities and the blot exposed to the luminol solution for 60 secs. After blotting dry using Whatmann 3mm paper (touching only the corner of the membrane) the membrane was exposed to X-ray film (Fuji) for varying exposure times (5 secs – 30 mins), dependent upon level of chemiluminescence and developed in an automatic X-ray film developer (X-ograph Compact X4)

3.5.5 Quantification of secondary carbonyl levels by spectrophotometry

Carbonyl levels were also analyzed according to the method of Levine *et al* (1994). Proteins in extracts (section 3.3) were first precipitated with ice-cold 10% (v/v) trichloroacetic acid (TCA) at 4°C for 10mins. The samples were then centrifuged at 15000g for 3 mins at room temperature. The protein pellet was re-suspended in 0.5ml 10 mM DNPH) in 2M HCl. Samples were then vortexed repeatedly (5 minute intervals) at room temperature for 1 hour. The samples were then re-precipitated by addition of 0.5ml of 20% TCA and centrifuged (15000g x 3 min, room temp). The pellet was washed with 1ml of ethanol-ethyl acetate (1:1 (v/v)) to remove residual free DNPH reagent, and allowed to stand for 10 min followed by a further centrifugation (15000g x 5min, room temperature) before discarding the supernatant. The ethanol-ethyl acetate washing and centrifugation steps were repeated three times. The protein pellet was then resuspended in 1ml 6M guanidine hydrochloride in 2mM phosphate buffer, pH2.3 and incubated for 30min at 37°C to dissolve the protein, followed by centrifugation to remove any residual particulate material (15000g x 3 min at room temperature). The absorbance of solutions was measured at 360nm using a 6M guanidine hydrochloride in 2mM phosphate buffer blank and secondary protein

carbonyls were calculated using a molar extinction coefficient of 22000 $M^{-1}cm^{-1}$. Protein concentration of the initial extracts was calculated from the absorbance at 280nm, allowing carbonyl content to be expressed as nmol carbonyl mg $protein^{-1}$.

3.6 Quantitative determination of active oxygen species production

Tetrazolium dyes were employed to detect superoxide radical generation by tuber cells. These compounds form characteristically coloured formazan dye products when reacted with radicals. Specifically 2,3-*bis*(2-methoxy-4-nitro-5-sulphophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT) was employed, chosen predominantly because the formazan dye products of XTT are soluble (formazan products are more commonly insoluble) and could thus be measured accurately, spectrophotometrically. The level of formazan dye product is then directly proportional to the amount of free radicals generated.

3.6.1 Tuber sample preparation

A 5mm diameter metal corer was used to excise 3mm deep cylindrical sections from the subdermal region of potato tubers at mechanically impacted sites and also at control sites. Each tissue section was washed extensively in qH_2O (3 changes, room temperature, with gentle mixing). Samples were then blotted dry again very gently to prevent mechanical stress. Tissues were then used immediately to measure radical generation by tetrazolium dye analysis.

3.6.2 Spectrophotometric quantification of superoxide production using XTT

Tuber tissue sections prepared as in section 3.6.1 were immediately placed in individual eppendorf tubes containing 200µl XTT solution (120µM XTT in 50mM phosphate buffer pH8.2) gently mixed and incubated in the dark at room temperature for 20 mins. The tissue sections were then removed and discarded and the assay solution briefly centrifuged (15000g x 30 seconds, room temperature) to remove any particulate material. The absorbance of the supernatant was then read at 450nm against an XTT-blank. Nanomoles of superoxide generated per gram fresh weight per minute was then calculated for each sample by using the molar extinction coefficient for XTT (23600 M^{-1} cm^{-1}) (Learmonth, personal communication).

3.7 Inhibitors of active oxygen species

To investigate the nature of the production of the active oxygen species further use was made of several inhibitors of active oxygen species production and action. Specifically the following were used - diphenylene iodonium chloride (DPI) which is a potent inhibitor of the NADPH oxidase complex – one of the two proposed mechanisms by which superoxide is generated. Superoxide dismutase (SOD) was also employed – this enzyme disproportionates superoxide into hydrogen peroxide and oxygen, thus having the effect of increasing hydrogen peroxide while not affecting generation of superoxide. In addition catalase was used, this enzyme decomposes hydrogen peroxide to water and oxygen but has no effect on superoxide levels.

A protocol was developed for exposing tuber sections to each of these inhibitors. Tubers were single impacted as described in section 3.1.1 followed immediately by bisection of the entire tuber through the point of impact. Each half was then

extensively washed in qH_2O (3 changes, room temperature) before being gently blotted dry. One half was placed in 5ml phosphate buffer (50mM pH7.2) and acted as the uninhibited control, the other half was placed in 5ml test solution (10μM DPI or 5μg catalase or 2.6μg SOD in 50mM phosphate buffer pH7.2) in a petri dish. Each tuber half was then wrapped in aluminium foil and incubated at 27°C for 1 to 48 hours (dependent upon the experiment) for bruise development.

3.8 Polyphenol oxidase and bruise pigment development

A key aspect of several experiments within this study was to observe the effect of various treatments upon bruise pigment development. Additionally a study was undertaken to ascertain what source of tyrosine could be utilised by polyphenol oxidase (PPO) in the production of melanin. To do this the catalysis of free tyrosine to melanin pigments by PPO was compared to catalysis of tyrosine-containing synthetic peptides by PPO. Finally the activity of PPO was studied in a variety of circumstances, this employed a catechol based assay initially used to assay PPO in lettuce developed whilst on industrial placement, and subsequently adapted for use in potatoes.

3.8.1 Polyphenol oxidase activity assay

This assay was initially used whilst on industrial placement with Dr S Clifford, HRI Wellesbourne, Warwicks. and was subsequently developed as follows for potato tubers. A quantified protein extract (sections 3.3 and 3.3.1) was prepared from mechanically impacted potato tuber (from both impact site and a control site). 975μl of 10mM catechol in 100mM phosphate buffer pH7.0 was added to 25μl of the protein extract followed by brief vortex mixing. The change in absorbance at 420nm was observed

every 5 seconds for 1 minute at room temperature, and the rate of change in absorbance per minute over the linear section of the graph calculated.

3.8.2 Catalysis of tyrosine (various sources) to melanin by PPO

Three synthetic peptides were used to evaluate the use of peptide bound tyrosine by PPO. The peptides were selected due to their varying levels of tyrosine coupled to other peptides known not to be involved in melanin synthesis. These peptides were abbreviated and composed as follows:

GAT631 (Glu : Ala : Tyr (6:3:1))

GAT111 (Glu : Ala : Tyr (1:1:1))

GA64 (Glu : Ala (6:4)) – control peptide with no tyrosine.

PPO (mushroom tyrosinase – Sigma) (25mg/ml in 50mM phosphate buffer pH7.3) was mixed with 2.5% (v/v) of either 10μg/ml L-tyrosine, 100μg/ml GAT631, 30μg/ml GAT111 or 50μg/ml GA64 (control), all in 50mM phosphate buffer pH7.3). Melanin formation was monitored by measuring the absorbance change at 475nm hourly for 24 hours, compared to a PPO (25mg/ml in 50mM phosphate buffer pH7.3) blank.

3.9 The effect of metal ions on blackspot.

Over several decades the involvement of various metal ions in modulating blackspot in potatoes has been investigated by several groups. Having investigated the role of active oxygen species on blackspot, a series of experiments were undertaken to investigate both the direct effect of metal ions on melanin formation and also the effect of metal ions on levels of secondary carbonyl accumulation in proteins (a marker for

damage caused by superoxide radicals). 8 metals were selected which had previously been shown to have an effect upon blackspot – Ca, Cu, Fe, K, Mg, Mn, Na and Zn.

3.9.1 Exposure of potato tuber proteins to metal ions.

Potato tuber protein extracts were prepared as in section 3.3, the buffer including 1mM of either Ca, Fe, K, Mg, Mn, Na (chlorides) or Zn and Cu (sulphates), or combinations of metal ions. Melanin production was monitored spectrophotometrically at 475nm hourly for 24 hours.

3.10 Materials

3.10.1 Plant Materials

Potato tubers, which had been grown and harvested to minimise mechanical stress by ADAS were supplied by the British Potato Council through the Sutton Bridge Experimental Unit, Sutton Bridge, Lincolnshire, UK (SBEU). Varieties supplied were Record, Cara, Russet Burbank, Maris Piper, Pentland Dell, Saturna and King Edward. Material was grown at two controlled sites, Arthur Rickwood and Terrington St Clement (appendix 1). Tubers were stored under optimal storage conditions to prevent sprouting and minimise changes in metabolic conditions (10°C, low light) at SBEU. Transport of tuber material from SBEU to Durham was carried out by manually packing tubers in pearlite or vermiculite to prevent mechanical impacts during transport. Other supplementary supplies of potato tuber material were sourced from various local commercial outlets and were used only for initial development and optimisation work, all experiments ultimately were carried out on SBEU material.

3.10.2 Reagents

Chemical reagents were of analytical grade or better and were supplied by Sigma (Poole, UK) except those listed below.

- BDH. Ltd (Leicestershire, UK) – acetic acid, hydrochloric acid, tyrosine, methanol
- Promega (Southampton, UK) – mid-range protein molecular weight markers
- Bio-Rad (Hemel Hempstead, UK) – Bradford reagent, ammonium persulphate
- National Diagnostics (Hull, UK) – Protogel (30% acrylamide / bis-acrylamide [37.5:1])
- Oncor [Intergen] (France) – OxyBlot kit.
- Boehringer-Mannheim (Lewes, East Sussex, UK) – POD Chemiluminescence reagent (peroxidase-linked detection systems)

All solutions were made with either distilled or purified distilled water (Elgastat, UHQPS, Elga Ltd, Buckinghamshire, UK).

3.10.3 Consumables

Plasticware was obtained from Greiner Ltd. Glassware was washed prior to use using proprietary detergents and thoroughly rinsed in distilled water. Nitrocellulose membranes were supplied by Schleicher & Schull (Dassel, Germany). X-Ray film (RX type) was supplied by Fuji, Tokyo, Japan.

3.10.4 Protein Analysis Equipment

SDS-PAGE was peformed using a Biorad Mini-Protean II system (Bio-Rad, Hertfordshire, UK). Western Blotting was carried out using a Biorad Mini-Protean II Blotting kit (Bio-Rad, Hertfordshire, UK). Spectrophotometry was carried out using a Beckmann D7500 diode array spectrophotometer.

3.10.5 Storage Facilities

Tubers were stored at Durham at 5°C in darkness to preserve dormancy and to inhibit greening. No use was made of anti-sprouting chemicals.

3.10.6 Computer software / hardware utilised.

Gel images were scanned using a Hewlett Packard ScanJet 11cx. Graphical and statistical analyses were performed using GraphPad Prism v3.0 (GraphPad Software).

RESULTS AND DISCUSSION

4. The involvement of active oxygen species in blackspot bruising

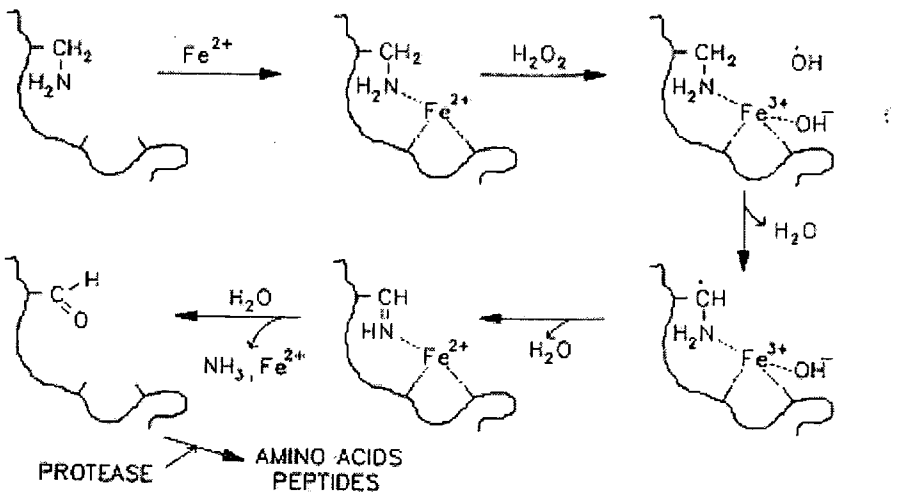
4.1 Introduction

Active oxygen species have been implicated in an ever expanding range of biological processes especially related to cellular stress for example in disease, cancer, ageing and in pathogen challenge. It was therefore an early and fundamental aspect of this project to ascertain whether, in response to mechanical impact, potato tubers also generate active oxygen species.

Prior to the present project no published work existed relating to active oxygen species generated following mechanical impact in potato tubers, though a small number of groups had been able to demonstrate the production of hydrogen peroxide in agitated (or mechanically stressed) cells in suspension culture (Collen and Pedersen, 1994, Yahraus *et al.*, 1995). It was therefore necessary to adapt techniques developed in other systems to study potato mechanical impact and to utilise new techniques as they became available during the course of the project in order to test the hypothesis that synthesis of blackspot bruise pigments is quantitatively determined by the level of generation of active oxygen species.

Upon exposure to the highly reactive active oxygen species, especially superoxide radicals, secondary carbonyl groups are introduced into amino acid residues in peptides (fig 4.1). Several amino acid residues can be modified by superoxide radicals : histidine, arginine, lysine, proline, methionine and cysteine (Levine, 1990). Especially reactive amongst these residues are arginine, proline and lysine which consistently show high susceptibility to superoxide modification. The presence of radical-generated

Figure 4.1 : Proposed mechanism for formation of carbonyl groups by exposure to active oxygen species. Adapted from Stadtman, 1993



carbonyl groups in proteins permits quantitative and qualitative assays to be developed for their detection.

As described in section 3.5.2 a chemical assay based upon dinitrophenylhydrazine (DNPH) was used to determine levels of secondary carbonyl modification. A convenient kit (Oncor OxyBlot) was available on the market which utilised DNPH, which can conjugate with secondary carbonyl groups to form dinitrophenylhydrazone derivatives. These DNP-hydrazone derivatives could then be detected using anti-DNP antibodies (Sigma-Aldrich). Thus after SDS-PAGE and Western Blotting proteins modified with carbonyl groups introduced by exposure to superoxide radicals could be localised by antibody probing. The kit, although producing some good results initially, proved to be very unreliable in its reproducibility. Subsequently several kit components were exchanged for self-prepared solutions with little improvement in the results. A further problem with this method was also encountered, due to a high concentration of salts in the derivatized proteins, normal running of SDS-PAGE analyses was disrupted. Desalting proved troublesome due to the small volumes involved. A change to a spectrophotometric method for quantifying oxidatively-induced carbonyl levels proved much more successful and reproducible (section 3.5.5). The results of the carbonyl assay work showed that in response to mechanical impact there was a significant and reproducible increase in oxidatively induced carbonyl levels in extracted tuber proteins. Consideration was then given as to whether generation of active oxygen species and especially free radicals could be detected directly. A recently developed technique utilising the tetrazolium dye 2,3-*bis*(2-methoxy-4-nitro-5-sulphophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT) was adapted for this purpose. Exposure of XTT to superoxide reduces the tetrazolium to a formazan dye product,

which could be quantitatively assayed spectrophotometrically. XTT was particularly applicable due to its high reactivity with superoxide to produce a formazan product soluble in aqueous solution (and thus measurable spectrophotometrically) (Sutherland and Learmonth, 1997). The assay developed to measure free radical generation did indeed show that tuber cells produced superoxide in response to physical impact.

Results from the XTT experiments gave an accurate time course of the production of superoxide following mechanical impact and led to the consideration as to whether a causal relationship could be established between superoxide production and pigment formation in blackspot bruising. The use of the NADPH-oxidase inhibitor diphenylene iodonium chloride (DPI) and the free radical scavenging enzymes superoxide dismutase (SOD) and catalase (CAT) enabled a partial elucidation of the link between superoxide production, active oxygen species and blackspot.

4.2 Results

4.2.1 Tuber varieties : susceptibility to blackspot.

A significant level of variability in properties exists between potato varieties caused by both biochemical-genetic and environmental factors, this is also reflected in blackspot bruise susceptibility. As stated in the introduction (section 1.2.1) variation in susceptibility to blackspot ranges from highly susceptible through to highly resistant. Seven potato varieties were used at various times during this work: Russet Burbank, Saturna, Record, Pentland Dell, Cara, King Edward and Maris Piper. Published literature was available from the Scottish Agricultural College reporting the bruise susceptibilities of each of these varieties, since environmental influence affects bruising actual bruise susceptibility of each variety was assessed prior to each variety being used

(table 4.1). Blackspot bruise index was calculated by measuring mean bruise depth, width and intensity in a sample of 25 tubers from each variety. The results were adjusted to a scale from 0 (complete resistance to mechanical impact) to 10 (very high susceptibility to mechanical impact – section 3.2). Tubers were regularly checked in this impact assay to ensure any variability change in the bruise indeces was detected.. This analysis confirmed that potato varieties employed in subsequent analyses were of a broad range of susceptibilities to blackspot bruise.

4.2.2 Analysis of secondary carbonyl level by immunoblotting

As part of a wider study being undertaken at the time into many aspects of blackspot bruising, attention turned towards the potential involvement of active oxygen species in the mechanical impact pathway. Several groups had presented work outlining the production of active oxygen species in response to many cellular phenomena, including by mechanical agitation of suspension cultures, which caused a transient increase in hydrogen peroxide levels to be observed.

As a consequence of exposure to superoxide free radicals proteins exhibit a characteristic chemical alteration, in which secondary carbonyl residues are introduced. A commonly adopted procedure for the detection of these oxidatively induced secondary carbonyl structures was employed – dinitrophenylhydrazine (DNPH) reacts with the carbonyl residues and covalently forms a dinitropheylhydrazone derivative. The hydrazone derivative can be assayed by several methods, including spectroscopy and immunoblotting.

Table 4.1 : Bruise indices of varieties used in this programme.

Potato varieties used in this programme with associated blackspot bruise indices as calculated prior to experimental use. Bruise index was calculated according to section 3.2.

* Blackspot bruise index is calculated from the mean bruise depth, width and intensity and was adjusted into the range:

0 = Completely resistant to blackspot *to* 10 = Very highly susceptible to blackspot.

Variety	Blackspot bruise index *	Relative blackspot susceptibility
Russet Burbank	9.2	Very High
Saturna	7.9	High
Record	5.9	Medium
Pentland Dell	5.5	Medium
Cara	5.5	Medium
King Edward	4.0	Low
Maris Piper	3.1	Very Low

Standard, modified and derivatised proteins were prepared for controls on SDS-PAGE analyses to confirm correct functioning of the detection system. Bovine serum albumin (BSA) and lysozyme were artificially exposed to superoxide free radicals, generated by the reduction of ferric chloride by ascorbate (section 3.5.1), and were subsequently derivatised with DNPH (section 3.5.2). The detection system was checked using the two derivatised proteins which were electrophoresed using SDS-PAGE, followed by Western blotting (sections 3.4 ; 3.5.3) and probing using anti-DNPHydrazone antibodies (section 3.5.4). The two protein samples were probed using a chemiluminescence detection system (Boehringer Mannheim kit based on luminol) and exposed to X-ray film (section 3.5.4). Polypeptides binding anti-DNP antibody showed up as dark bands corresponding to proteins containing secondary carbonyl structures (figure 4.2). This showed that freshly sourced BSA had no visible oxidative protein modification, whereas the sample exposed to superoxide showed significant levels of secondary carbonyl accumulation. The unmodified lysozyme (which was several years old), showed a significant level of modification in the sample not exposed to superoxide radicals, but showed an enhanced level in the sample exposed to free radicals. The observation that old lysozyme already had a high level of oxidative modification could potentially be explained by the age of the sample – as proteins age prolonged exposure to naturally occurring active oxygen species increases and consequently oxidative modifications to the protein structure are likely to increase.

4.2.3 Oxidative modification of tuber proteins

The potato cultivar Record was selected to test for oxidatively induced protein changes after mechanical impact (Record was the most bruise susceptible variety available at the time of these experiments). Record tubers were impacted, the proteins extracted

Figure 4.2 : Immunoblot analysis using anti-DNPH antibodies against standard proteins. Figure shows results using BSA and lysozyme artificially modified by treatment with superoxide radicals.

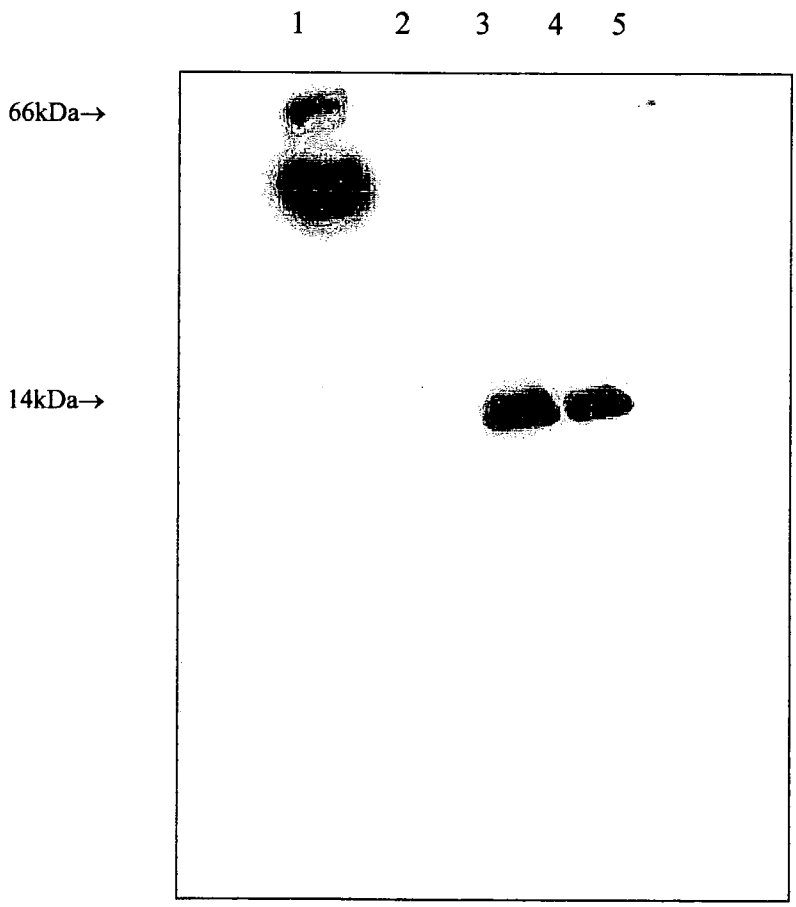
Key: 1 – BSA exposed to superoxide radicals (10μg)

2 – BSA control (10μg)

3 – Lysozyme exposed to superoxide radicals (10μg)

4 – Lysozyme control (10μg)

5 – Commercially obtained BSA exposed to superoxide (3μg)



and quantified (sections 3.1.1, 3.3, 3.3.1). The proteins were derivatised and immunoblotted using the procedure described in section 4.2.3 for lysozyme (figure 4.3). This preliminary analysis clearly showed that in impacted tissue, a substantial level of secondary carbonyls were detected, and in non-impacted tissues almost no secondary carbonyls were noted. This suggested that tuber proteins from the area of mechanical impact had been exposed to a significant quantity of superoxide free radicals. Trace quantities of carbonyls were detected in several replicate samples from the non-impacted tissue (data not shown). This was attributed to proteins modified by free radicals produced by normal cellular reactions – for example during mitochondrial respiration. This result was highly significant as it was the first demonstration that in response to mechanical impact in potatoes superoxide free radicals were released as a response. This finding was to form the basis for much of the subsequent work undertaken in this project.

4.2.4 Variation in oxidative modification between potato varieties

Each of eight potato varieties were mechanically impacted and assayed for oxidatively modified proteins to ascertain whether any differences could be detected between potato varieties with varying susceptibilities to blackspot bruising (figure 4.4). The results showed reproducible increases in the level of oxidatively modified proteins broadly corresponding to the increasing susceptibility to blackspot bruise. This suggested that in tandem with increasing levels of pigment production there was potentially a corresponding increase in the level of superoxide generation.

Figure 4.3 : **Immunoblot analysis of Record tuber proteins.** Impacted and non-impacted samples from cv.Record using anti-DNPH antibodies to detect carbonyl modifications arising due to oxidative damage. The blot was exposed for 3 minutes and prepared as described in sections 3.5.2 to 3.5.4.

Key: 1 – Modified ovalbumin and carbonic from OxyBlot kit (15ng each)

2 – Control non-impacted Record (not exposed to DNPH) (7μg)

3 – Non-impacted Record (exposed to DNPH) (7μg)

4 – Control impacted Record (not exposed to DNPH) (7μg)

5 – Impacted Record (exposed to DNPH) (7μg)

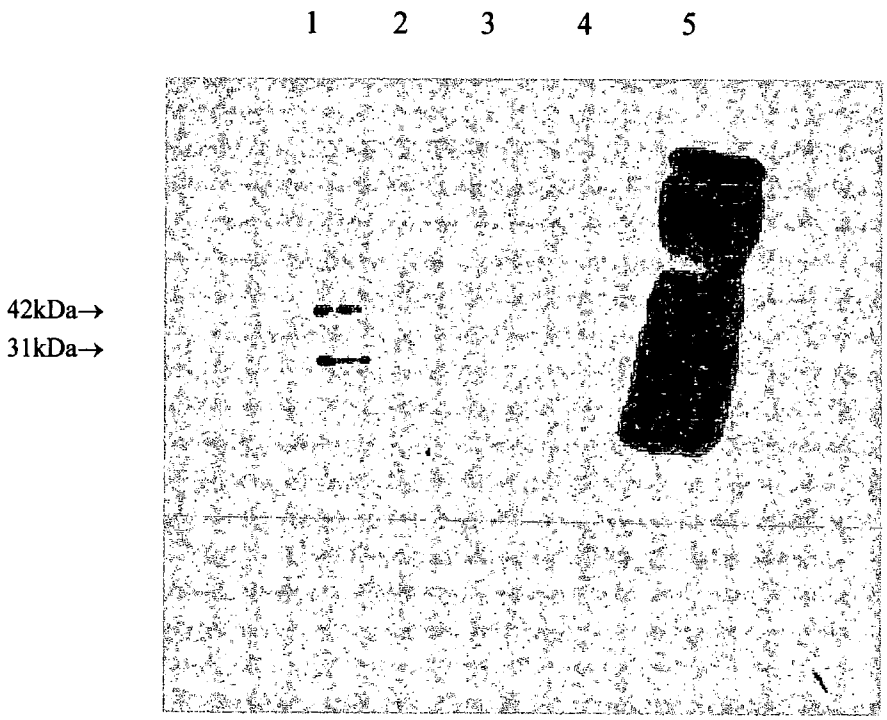


Figure 4.4 : Immunoblot of proteins from impacted tubers. Analysis of eight potato varieties with varying degrees of susceptibility to blackspot probed with anti-DNPH antibodies to detect oxidatively modified proteins. Blot was exposed for 30 seconds, and was carried out as described in sections 3.5.2 to 3.5.4). 7μg of each protein was loaded. Susceptibility to blackspot bruise in parentheses. The 39.9 kDa band marked corresponds to patatin, the major storage protein of potato tubers.

Key : 1 – Maris Piper (very low)

2 – King Edward (low)

3 – Cara (medium)

4 – Pentland Dell (medium)

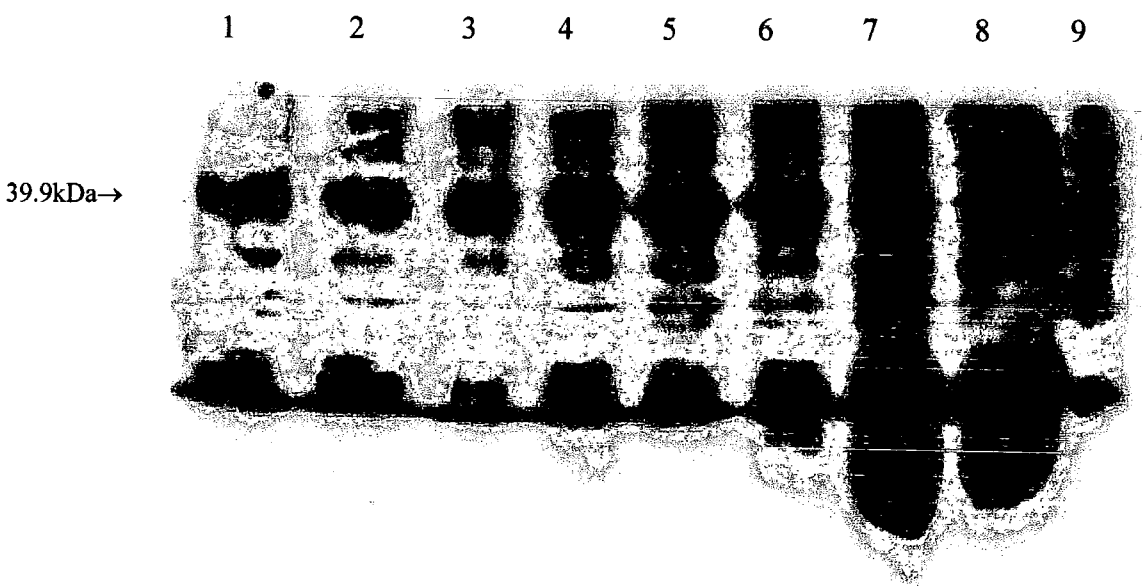
5 – Desiree (medium)

6 – Record (medium)

7 – Saturna (high)

8 – Russet Burbank (very high)

9 – Standard oxidatively modified proteins (OxyBlot kit)



4.2.5 Appraisal of the immunoblotting methodology

The immunoblotting procedure for assaying oxidatively induced carbonyl modifications had several major drawbacks:

1. The method was unable to accurately quantify the levels of carbonyls present at least using the analytical system used here (SDS-PAGE)

2. The method produced inferior qualitative results, making conclusions less accurate and subsequent publication difficult.

3. The method was unreliable. The method was labour intensive but was not technically difficult and as a consequence was slow.

4. The method, employed initially a commercial kit (Oncor OxyBlot), which unfortunately suffered from excessive unreliability of components, which could not be entirely resolved by sourcing materials elsewhere.

Due to these reasons the use of the immunoblotting assay for secondary carbonyls was discontinued and a spectrophotometric method for quantifying DNPHydrazone carbonyls adopted.

4.2.6 Quantitative assay for carbonyl levels in proteins

The ability to quantify secondary carbonyl levels in a spectrophotometric assay (section 3.5.5) enabled much more accurate comparisons to be drawn between potato varieties susceptible and resistant to blackspot bruise (Levine *et al.*, 1994). Five varieties,

representing the range of blackspot susceptibilities, were assayed by this method and the carbonyl levels quantified (figure 4.5). Again a high degree of carbonyl modification was seen in proteins from impacted tissue from the highly susceptible varieties, compared to the non-impacted tissues. A relatively low level of carbonyl accumulation was seen in all varieties in the non-impacted tissues, this background level was again presumably the effect of normal metabolically generated free radicals on tuber proteins. Again the level of carbonyl modification was proportional to the susceptibility of the varieties to blackspot bruise – the mean carbonyl level was plotted against the bruise indices for each variety (section 4.1) to assess any correlation between carbonyl accumulation and blackspot bruise (figure 4.6). The Pearson R value of correlation for these two factors was calculated to be 0.972, an extremely high level of correlation.

4.2.7 Detection of superoxide generation following impact using XTT

Although the presence of secondary carbonyl groups are highly indicative of exposure to superoxide radicals, a method was developed to directly measure the level of superoxide generation from tuber tissues. The new method utilised one of a class of compounds containing tetrazolium functional groups. The tetrazolium group readily reacts with superoxide free radicals and forms a formazan product which is invariably a different colour to the dye itself (figure 4.7). In particular XTT was selected as the formazan product of XTT was unusual in being soluble in water, and could therefore be assayed spectrophotometrically using a method similar to that developed by Sutherland & Learmonth (1997). The methodology was jointly developed with Dr S Doherty for use in the potato tuber system (section 3.6.2).

Figure 4.5 : Quantitative assay of accumulation of carbonyl levels in five potato varieties.

Carbonyl levels from five varieties (impacted and non-impacted tissue) were compared. Assay was carried out as described in section 3.5.5 – proteins were extracted and the absorbance read at 360nm. Error bars are SD of mean, experiment was carried out in triplicate.

Bruise index given in paretheses (0 – highly blackspot resistant → 10 highly blackspot susceptible)

Key : RB – Russet Burbank (9.2)

SA – Saturna (7.9)

CA – Cara (5.5)

KE – King Edward (4.0)

MP – Maris Piper (3.1)

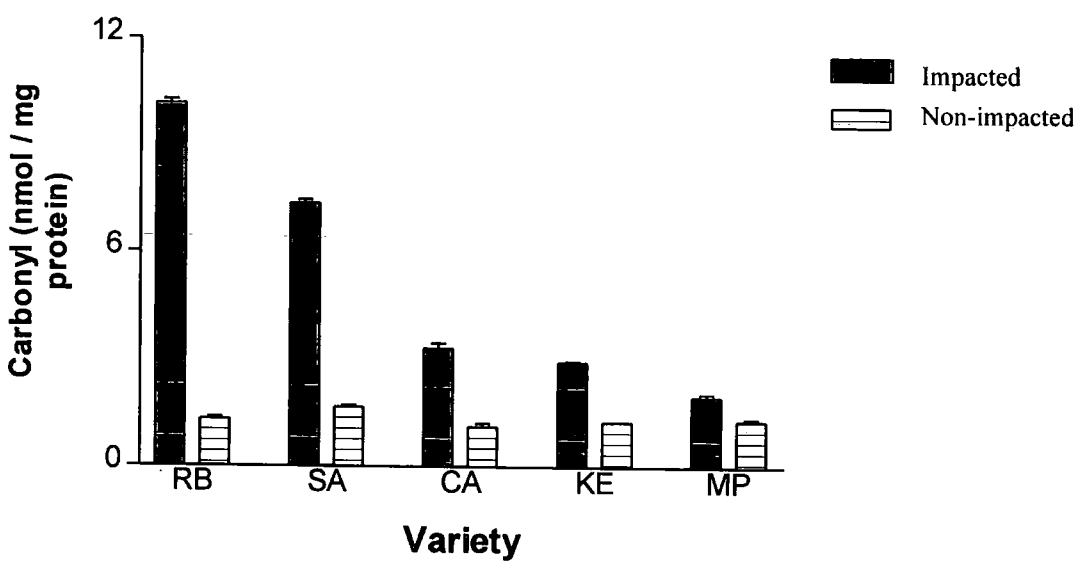


Figure 4.6 : Correlation between carbonyl levels and tuber bruise susceptibility . The Pearson R value of correlation in 0.972 indicates a very high level of correlation. Varieties used to establish this correlation were Russet Burbank ; Saturna ; Cara ; King Edward and Maris Piper (described in section 4.2.6). The data were plotted and statistically analysed using GraphPad Prism software (v3.0).

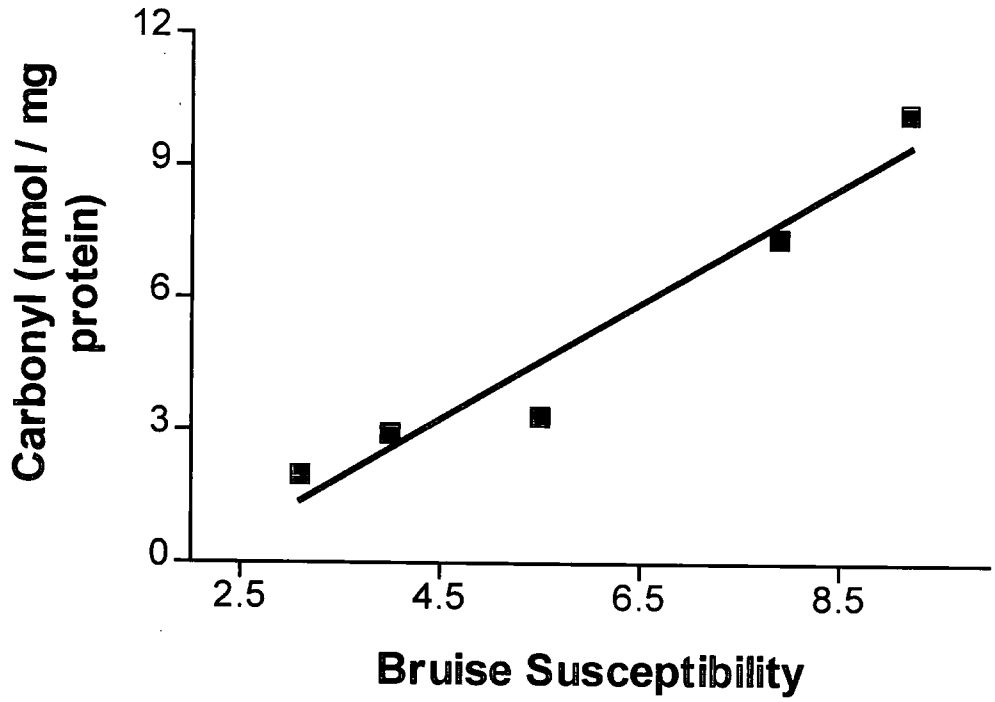
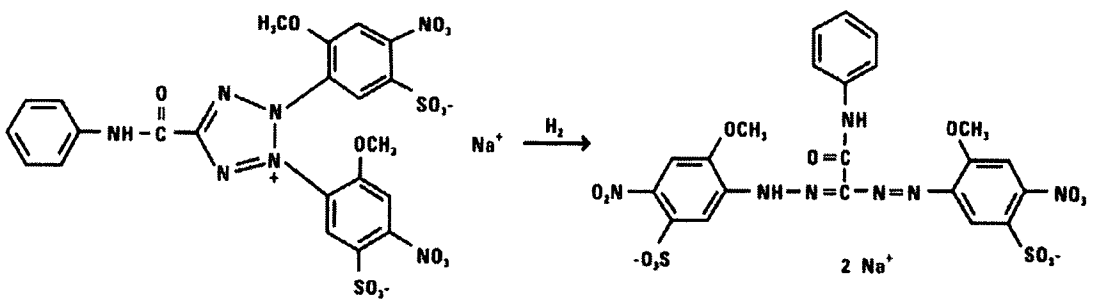


Figure 4.7 : Structure of XTT and formazan product. From Sutherland and Learmonth 1997



XTT
MW. 673.5

XTT Formazan

Each of five tuber varieties (representing the full range of susceptibilities to blackspot) was assayed using the XTT protocol (section 3.6.2). Superoxide levels were assayed in tubers from each variety hourly over the 7 hours post mechanical impact (figure 4.8 to 4.12). These results confirmed the previous carbonyl assays, by demonstrating that superoxide radicals were being produced by potato tubers at the site of mechanical impact albeit after a shorter period of time. The radicals were being produced rapidly (detectable within 2 hours of impact) and their generation was sustained for up to 5 hours. Again with increasing sensitivity to blackspot there was a corresponding reduction in the level of superoxide radicals being produced (table 4.2). These findings were rather at odds to those of Stevens and Davelaar 1997, who reported that all regulation of cellular responses was broken down after impact due to complete destruction of the cells in the vicinity of the impact. Here, however, an apparently controlled reaction was taking place, directly as a response to mechanical impact and localised to that site. This response was subsequently quantified and correlated with the bruise indices for the varieties tested (section 4.2.8).

During the time-course experiments a reproducible dip in superoxide production was observed in all varieties tested between 2-3 hours from time of impact (clearly demonstrated in figure 4.9). Interestingly this reduction in superoxide production mimicked that seen in the superoxide response to bacterial elicitation in plants. In that system the production of superoxide prior to the dip (primary phase) has been ascribed to be a non-specific biological reaction to any stress. The second phase (post 4 hours) however is known to be a specific reaction to the bacterial elicitation and involves gene activation (Lamb and Dixon 1997; Wojtaszek, 1997). The prospect that specific gene alteration may occur as a response to mechanical impact was of great significance as

Table 4.2 : Maximum superoxide generation in five potato varieties. Table summarises maximum level of superoxide generation four hours post-mechanical impact in different potato varieties compared with their blackspot bruise susceptibilities. The data are extrapolated from figures 4.7 to 4.11. Bruise index values relate to figures calculated and presented in table 4.1, 0 represents complete resistance to blackspot, 10 represents very high susceptibility to blackspot. The mean background level of superoxide generation was 0.91 nmol g^{-1} min^{-1} and represents control (non-impacted) levels of superoxide i.e. levels in normal tissues and not produced directly as a result of mechanical impact

Variety	Bruise Index (0-10)	Maximum superoxide production after subtraction of mean control level of superoxide (nmol g^{-1} min^{-1})
Russet Burbank	9.2	3.46
Saturna	7.9	2.20
Cara	5.5	1.91
King Edward	4.0	0.99
Maris Piper	3.1	0.41

Figure 4.8 : **Superoxide generation by cv. Russet Burbank.** Figure shows production of superoxide radicals in Russet Burbank potatoes, quantified by the use of XTT as described in section 4.2.7. Russet Burbank has very high susceptibility to blackspot bruise. Error bars are SD of mean, experiment was carried out in triplicate. Error bars not shown are hidden by symbol.

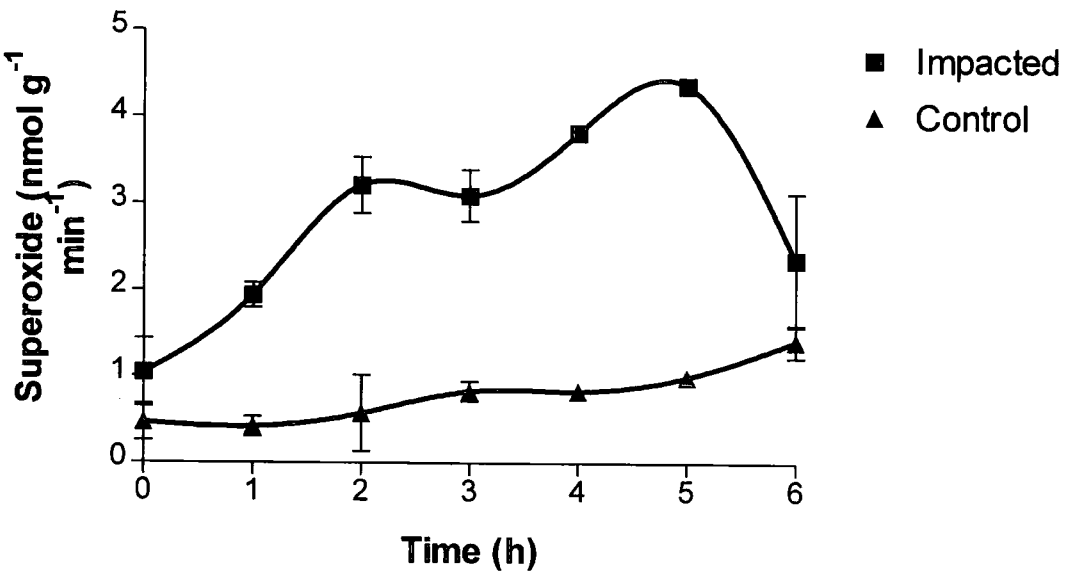


Figure 4.9 : Superoxide generation by cv. Saturna. Figure shows production of superoxide radicals in Saturna potatoes, quantified by the use of XTT as described in section 4.2.7. Saturna has high susceptibility to blackspot bruise. Error bars are SD of mean, experiment was carried out in triplicate. Error bars not shown are hidden by symbol.

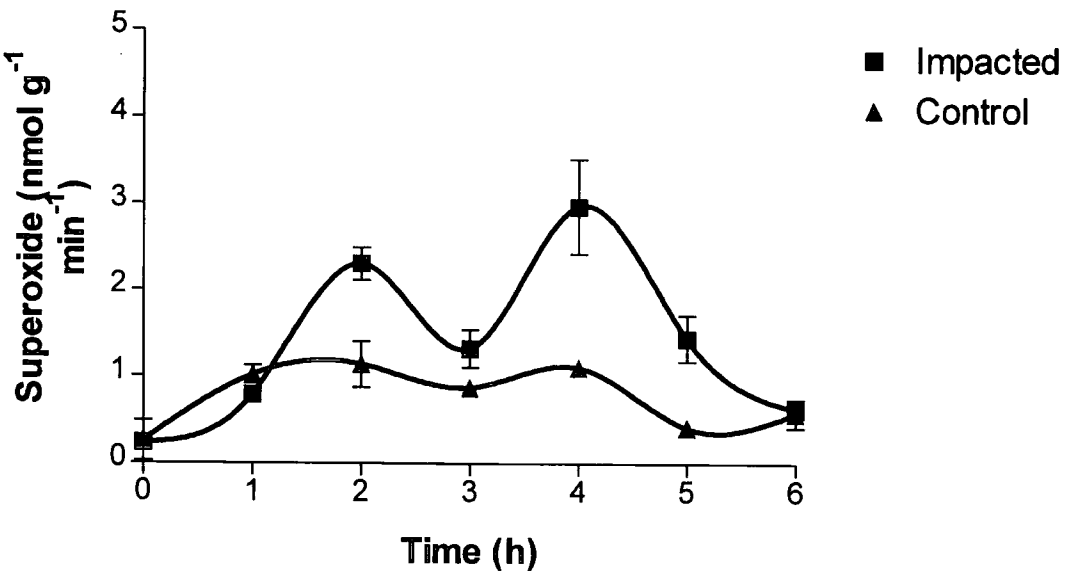


Figure 4.10 : Superoxide generation by cv. Cara. Figure shows production of superoxide radicals in Cara potatoes, quantified by the use of XTT as described in section 4.2.7. Cara has medium susceptibility to blackspot bruise. Error bars are SD of mean, experiment was carried out in triplicate. Error bars not shown are hidden by symbol.

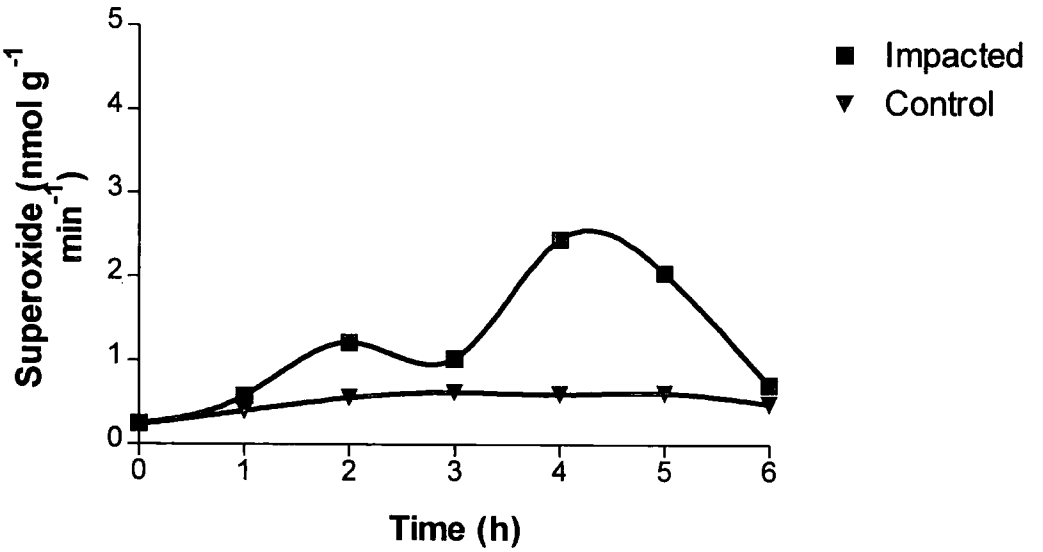


Figure 4.11 : **Superoxide generation by cv. King Edward.** Figure shows production of superoxide radicals in King Edward potatoes, quantified by the use of XTT as described in section 4.2.7. King Edward has low susceptibility to blackspot bruise. Error bars are SD of mean, experiment was carried out in triplicate. Error bars not shown are hidden by symbol.

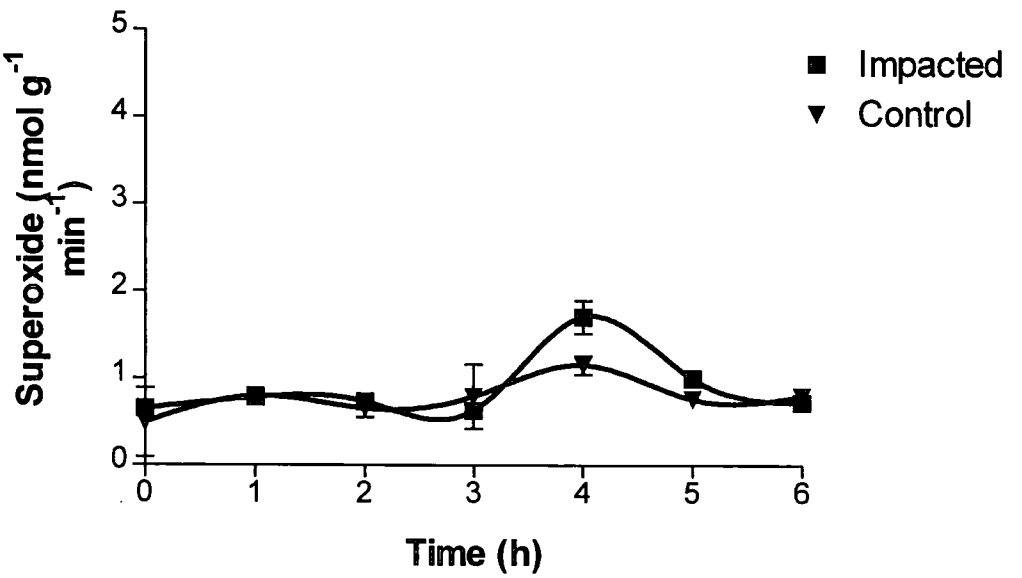
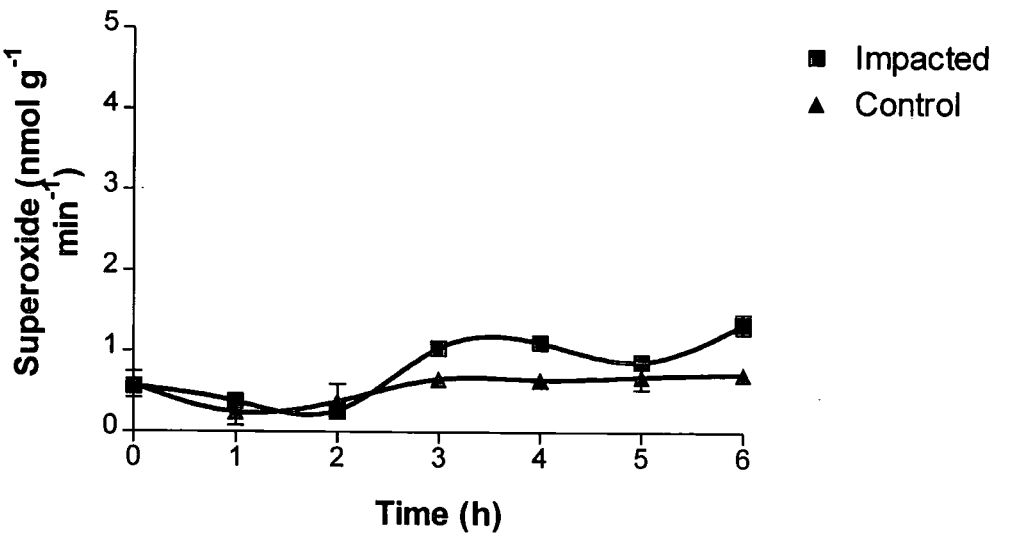


Figure 4.12 : Superoxide generation by cv. Maris Piper. Figure shows production of superoxide radicals in Maris Piper potatoes, quantified by the use of XTT as described in section 4.2.7. Maris Piper has very low susceptibility to blackspot bruise. Error bars are SD of mean, experiment was carried out in triplicate. Error bars not shown are hidden by symbol.



the bacterial elicitor-plant interaction was believed to have a receptor-ligand type of interaction between the elicitor and plant. Obviously, in the potato mechanical impact system, no pathogen derived ligand was present.

4.2.8 Correlation between superoxide generation and blackspot bruise index

The Pearson R value of correlation between secondary carbonyl level and bruise index of 0.972 indicated a clear linear correlation between these two factors. A similar correlation plot between maximal superoxide generation using data from the same five varieties and bruise index (section 4.1) was constructed (figure 4.13). The calculated Pearson R value (R=0.9688) revealed a very high level of correlation between superoxide generation and blackspot bruise index and was reproducible to within +/- 0.03. This high level of correlation supported and confirmed the suggestion that secondary carbonyl levels noted in post impact tubers were caused directly by the action of a rapid and regulated release of superoxide, induced directly by mechanical impact.

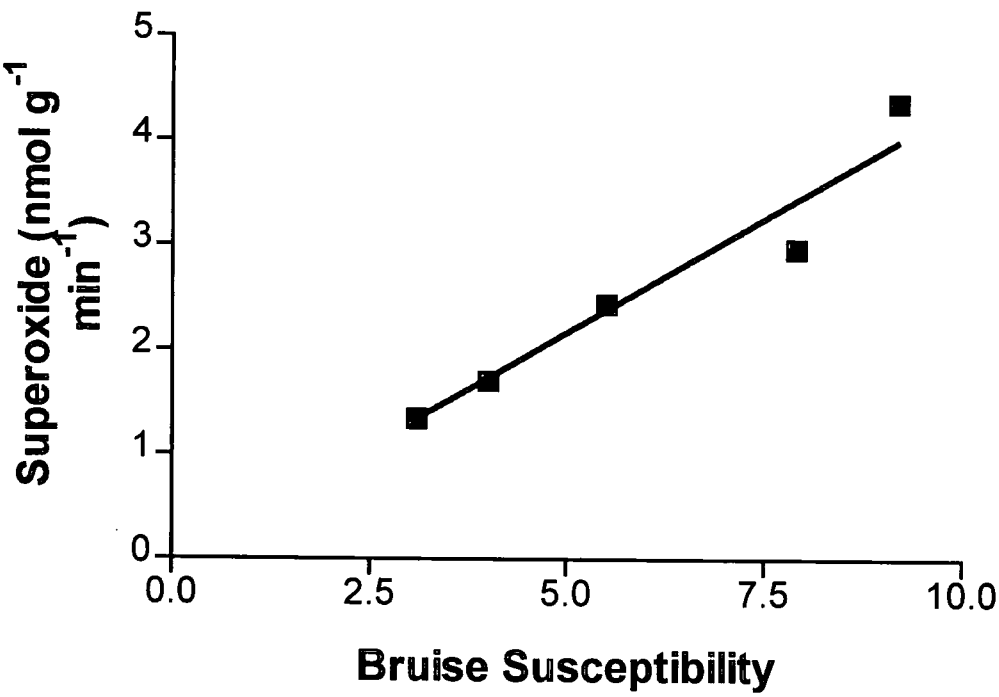
This high degree of correlation was considerably higher than any previous correlated factor and created the potential for the development of a novel methodology to predict blackspot susceptibility in tubers before harvest more accurately. This aspect was one of the major objectives of the project.

4.2.9 Comparison of superoxide at different regions of the tuber

Sabba and Dean (1994) noted that tubers exhibited a variability in their capacity to produce bruises dependent on the position of the impact on the tuber. They noted

Figure 4.13 : Correlation between maximum superoxide production following impact and tuber bruise susceptibility. The Pearson R value of correlation is 0.9688 indicating a very high level of correlation.

Varieties used were Russet Burbank ; Saturna ; Cara ; King Edward and Maris Piper for this experiment (described in section 4.2.8). Data plotted and statistically analysed using GraphPad Prism software (v3.0).



particularly that tubers bruise more readily and more extensively at the stolon end compared to the bud end.

As part of the testing of the hypothesis that radical generation was correlated with intensity of bruise produced samples of five varieties of potato were impacted at either the stolon or bud end and immediately assayed for superoxide production using the XTT assay after 5h (section 4.2.7). These data shown in figure 4.13 suggest that on average the bud end of the tuber shows a 35-50% lower generation of superoxide after 5 hours compared to the stolon end. This result compares favourably with the results of Sabba and Dean (1994) who reported a 40% reduction in bruising capacity at the bud end compared to the stolon end. Again the results in figure 4.14 suggest superoxide generation is directly correlated with bruise production.

4.2.10 Effect of inhibitors on superoxide production

The nature of the XTT assay, in which excised tuber cells in tissue sections are exposed to XTT in solution implies an extracellular generation of superoxide radicals. This is in agreement with the two alternate views of superoxide production in plants currently being suggested. Bolwell, 1998 proposes a pH dependent cell wall associated peroxidase, in which a transient rise in cellular pH (alkalinisation) causes induction of a cell wall peroxidase and subsequent superoxide generation. Alternatively several groups support the idea that a system very similar to that seen in the mammalian phagocytes exists in plants (Lamb and Dixon, 1997) in which activation of a trans-membrane NADPH dependent oxidase enzyme generates superoxide while reducing NADP to NADPH. Both methods would potentially direct superoxide radicals into the

Figure 4.14 : Superoxide generation at different points on the tuber. Figure shows comparison of maximum superoxide production 5h after impact at one of two impact points (bud end or stolon end) in five different varieties of potato. Experiment carried out as described in section 4.2.9. Error bars are SD of mean, experiment was carried out in triplicate.

Bruise indices in parentheses (0 – highly resistant to blackspot, 10 – highly susceptible to blackspot)

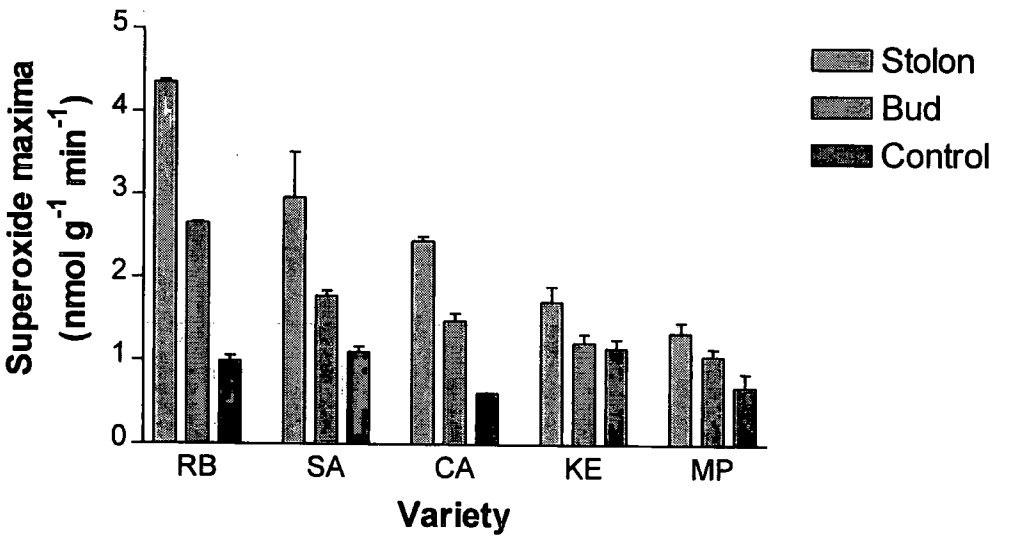
Key: RB – Russet Burbank (9.2)

SA – Saturna (7.9)

CA – Cara (5.5)

KE – King Edward (4.0)

MP – Maris Piper (3.1)

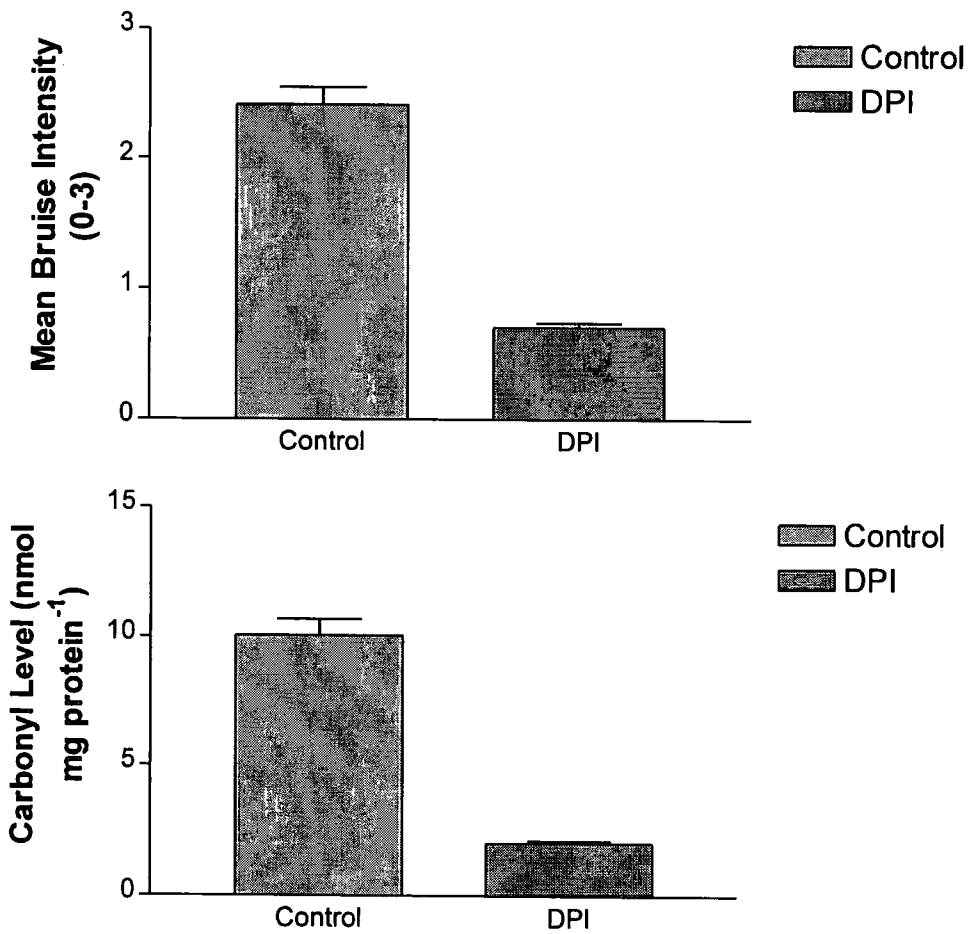


extracellular space. An experiment using a potent inhibitor of NADPH oxidase was developed – diphenylene iodonium chloride (DPI) (section 3.7)

Russet Burbank tubers were selected (because of their high susceptibility to blackspot bruising) and were impacted immediately followed by exposure to 6.6μmol/ml DPI and then assayed for bruise production (section 4.1) and secondary carbonyl levels in tuber proteins (section 3.5.5) (figure 4.15). In a joint series of experiments Mr A Brown (3rd year project student), conclusively demonstrated that although DPI is held to have an inhibitory effect on some oxidase enzymes, the key enzyme in the blackspot pathway – polyphenol oxidase – was not inhibited by DPI. The effect of the DPI was shown to significantly reduce the bruise index of Russet Burbank. This is normally a highly susceptible variety but in the presence of DPI gave the response of a highly resistant variety. Assays for secondary carbonyl levels were reduced to those seen in non-impacted samples. The conclusions from this were two fold 1. NADPH oxidase was responsible for superoxide generation and 2. DPI inhibited the superoxide generation and this was having a direct effect on pigment synthesis.

Together these results support the view that in potato tubers NADPH oxidase is largely responsible for the generation of superoxide radicals. The superoxide radicals interact with tuber proteins. The reduction in bruising also implies a direct link between superoxide generation and melanin synthesis.

Figure 4.15 : Effect of DPI on bruise intensity and carbonyl level. Figure demonstrates the effect of the NADPH Oxidase inhibitor diphenylene iodonium chloride (6.6 μmol/ml) on mean bruise intensity and accumulation of carbonyl groups, assayed as described in sections 3.2, 3.5.5 and 3.7. Experiments carried out using impacted tubers of cv. Russet Burbank. Error bars are SD of mean, experiment was carried out in triplicate.



4.2.11 The effect of free radical scavenging enzymes

Further indications of the involvement of active oxygen species were obtained using enzymes which remove specific active oxygen species. Two enzymes were chosen for these studies; superoxide dismutase and catalase.

Superoxide dismutase (SOD) disproportionates superoxide free radicals into hydrogen peroxide and oxygen (Streller and Wingsle, 1994). Thus generation of superoxide (as seen at mechanical impact in potato) the addition of SOD would sequester the radicals and dismutate them preventing reaction with XTT. When impacted sections of Russet Burbank tuber were exposed to 7nmol/ml SOD and assayed for bruise index and carbonyl accumulation (section 4.2.11) a substantial reduction in both bruise index and carbonyl levels were observed (figure 4.16) compared to the control sample. This lends further supporting evidence to the key role played by superoxide radicals in the bruise pigment forming process.

Catalase detoxifies hydrogen peroxide to water and oxygen (Willekens *et al.*, 1995), thus if present during the rapid release of superoxide it would have no effect upon the elevated superoxide levels, but would reduce the overall hydrogen peroxide level. When Russet Burbank tubers exposed to 61μmol/ml catalase were assayed for bruise index and secondary carbonyl level no significant difference was observed to that of the control sample (figure 4.17). This result, taken with the previous, in which the effect of SOD would be to increase hydrogen peroxide levels, suggests that hydrogen peroxide plays no part in blackspot bruising directly.

Figure 4.16 : Effect of superoxide dismutase on bruise intensity and carbonyl level. Figure shows the effect of the superoxide scavenging enzyme superoxide dismutase on mean bruise intensity and on carbonyl accumulation. Experiments were carried out as described in sections 3.2, 3.5.5 and 3.7. Experiments carried out using impacted tubers of cv. Russet Burbank. Error bars are SD of mean, experiment was carried out in triplicate.

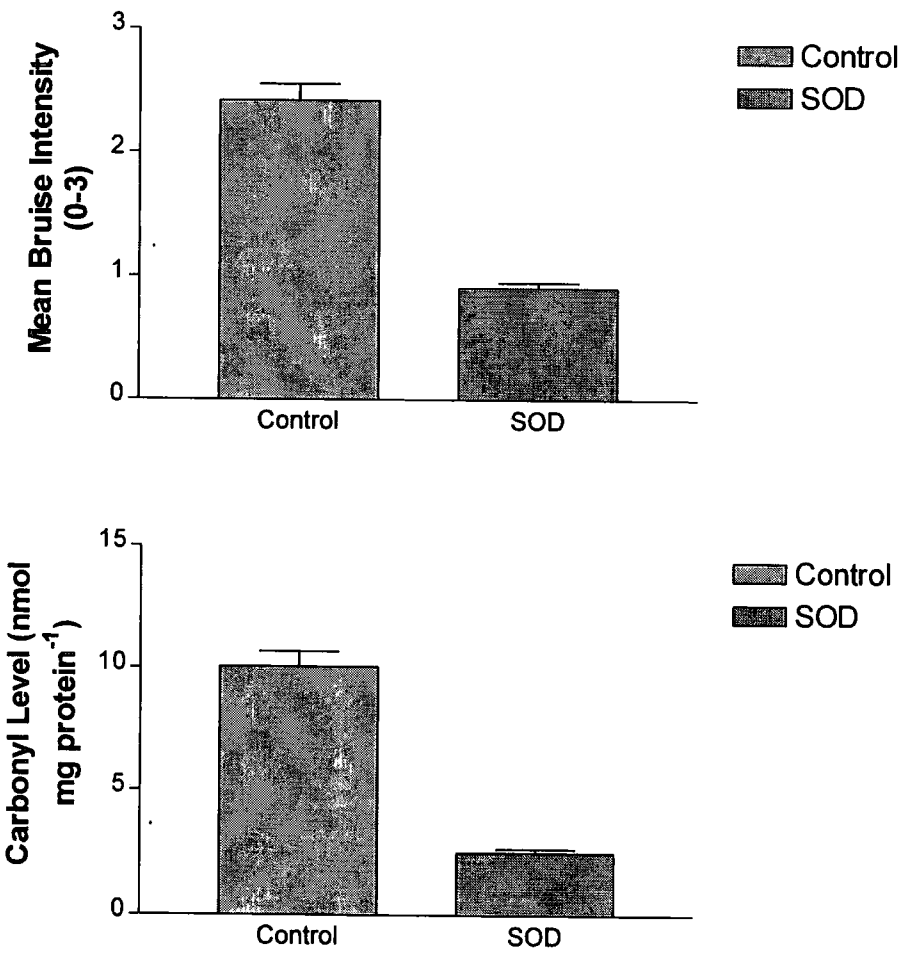
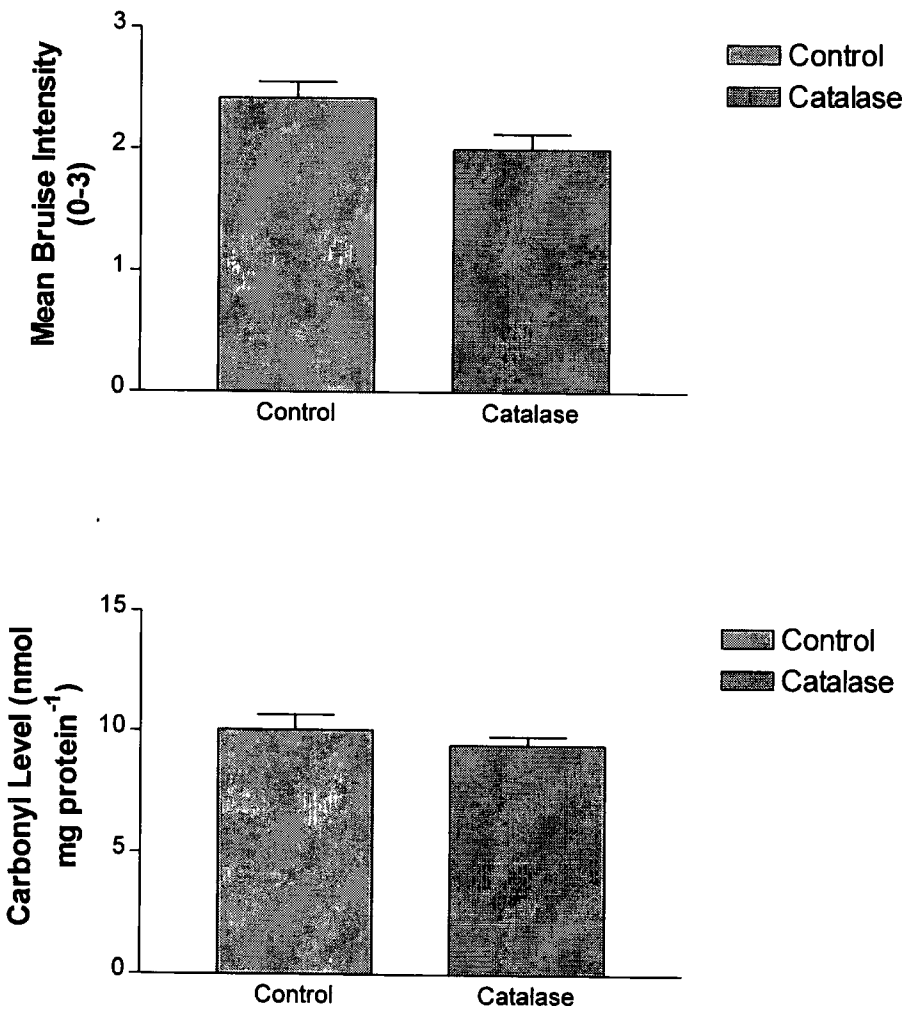


Figure 4.17 : Effect of catalase on bruise intensity and carbonyl level. Figure shows the effect of the hydrogen peroxide scavenging enzyme catalase on mean bruise intensity and on carbonyl accumulation. Experiments were carried out as described in sections 3.2, 3.5.5 and 3.7, using impacted tubers of cv. Russet Burbank. Error bars are SD of mean, experiment was carried out in triplicate.



4.3 Discussion

This section represents the identification and development of a novel series of findings within potato blackspot bruise research which potentially could have significant implications not only for the detection and inhibition of blackspot bruise but also in the further elucidation of the mechanism of pigment synthesis.

Although changes in protein composition could not be detected either pre or post-bruising significant changes were taking place to protein structure as revealed by assaying for oxidative modifications induced by superoxide radicals. Alterations to protein structure caused by exposure to oxidative damage were measured by assaying secondary carbonyl levels through the use of DNPH derivatisation, and is a widely used marker assay in animal systems for the diagnosis of diseases where neutrophil function is impaired (Levine *et al.*, 1994; Reznick and Packer, 1994; Burcham and Kuhan, 1996). However recent evidence points to a more varied origin for such modifications (Adams *et al.* 2001). As well as thorough exposure to superoxide free radicals, carbonyl groups may also be introduced into protein structures by other oxidative compounds, including peroxynitrite, and by other non-oxidative processes including incubation with various aldehydes. Peroxynitrite is of interest because it is produced naturally by the interaction of superoxide free radicals (derived from molecular oxygen) and nitric oxide radicals (derived from molecular nitrogen). The superoxide and nitric oxide radicals combine to form the very highly reactive peroxynitrite $OONO^-$. This is of special note as peroxynitrite has now been demonstrated to be generated in tubers after mechanical impact (work carried out subsequent to this project in this group). In this way either superoxide or peroxynitrite could be postulated to be responsible for the introduction of carbonyl side-groups to proteins. In either case the

introduction of carbonyl groups is presumably detrimental to the protein, with reports of loss of catalytic function in enzymes, increased sensitivity to denaturation and increased susceptibility to proteolysis all being suggested changes induced by increasing carbonyl content in animal proteins (Beckman and Ames, 1998; Dean *et al.* 1997; Stadtman *et al.* 1992; Grune *et al.*, 1997). However the introduction of secondary groups to protein structures in plants alters their nutritional quality and has been demonstrated to make them less nutritionally valuable and digestable to potential consumers (Freidman, 1997). In this way the introduction of carbonyl structures to proteins in the vicinity of the mechanical impact may be envisaged as a specific response, if, for example, the potato had initiated a wounding type reaction as a response to mechanical impact.

Work utilising the tetrazolium XTT, clearly demonstrated the generation of superoxide free radicals as a response to mechanical impact. This is the first demonstration of superoxide being produced directly as a response to mechanical impact in plants, previous work into mechanical stress implying radical intermediates has only demonstrated hydrogen peroxide production (Collen and Pedersen, 1994, Yahraus *et al.*, 1995). This is a key finding as free radicals have been implicated in a wide range of biological systems and opens up a new area for investigation within blackspot bruising.

Superoxide generation following mechanical impact displays a regular and reproducible pattern of synthesis, with peaks at 1-2 hours (following impact) at approximately 75% of maximal production followed by a lag period of 1-2 hours in which production falls to levels similar to that noted from routine cellular processes.

After the lag period a 3-4 hour second peak of superoxide occurs in which production reaches maximal levels before returning to basal levels. This two phase production is of significance as it very closely mimicks the superoxide production profile seen after plant cells are challenged by a microbial elicitor (Lamb and Dixon, 1997). In the microbial elicitor system the two phases are believed to have differing origins. The first phase is non-specific and occurs when any biological material undergoes any form of stress (indeed it is this phase which is seen during mechanical agitation of suspension cells). The second phase is reported to be a specific biological reaction initiated by a receptor-ligand complex involving both the elicitor and the plant. Extrapolating this model to the mechanical impact system poses a fundamental problem – what causes the second phase of superoxide production become initiated? The first phase (non-specific) would be expected however a receptor-ligand type of interaction would initially seem improbable since no pathogen is involved. However, initial results from work recently carried out in this group indicate a role for self-generated fragments. Similar work on plant cell walls by Fry (1998) suggests oligosaccharide fragments are produced by oxidative scission and it therefore may be proposed as the basis for self-initiation of the oxidative burst in this system. This work is on-going.

When considering why the oxidative burst takes place in this system it must be considered that either the pathogen defence responses or the wounding responses might be activated. Both pathways involve the initial production of superoxide (Lamb and Dixon, 1997; Bergey *et al.*, 1996; Chai and Doke, 1987) and both might be activated by the plant if it were to 'misread' the mechanical impact as either pathogen challenge or an insect attack. Credence is given to this notion when it is considered that a response

to mechanical impact would not be necessary, from an evolutionary point of view, given the crop grows underground where the chance of mechanical impact is slight.

A role for superoxide in the metabolism of melanin should also be considered. Recent work in animal studies has demonstrated a key role for superoxide radicals as a co-substrate for polyphenol oxidase (Valverde *et al*, 1996). This group demonstrated that where superoxide radicals and molecular oxygen were both present, polyphenol oxidase utilised superoxide preferentially. Furthermore, when using superoxide the rate of formation of melanin was enhanced considerably. In this way, melanocytes (which were the basis of the Valverde study) were postulated to have a novel anti-oxidant defence mechanism. Melanin itself can protect cells against free radicals as it is a potent free radical sink (Zughaeir *et al.* 1999; Bustamante *et al.*, 1993; Riley, 1997; Mosca *et al.*, 1998). Given this property of the pigment, and the fact that it is reported to be primarily deposited around the edges of cells and membranes (Partington *et al.*, 1999) it could also be argued that melanin, and potentially other polyphenols synthesised as a result of mechanical impact (Stevens *et al.*, 1998) could have a primary function to defend the outer cell membrane and inner cell wall against free radical attack by coating delicate surfaces and scavenging free radicals. This is further supported by the work suggesting that the origin of superoxide radicals in this system is the transmembrane NADPH oxidase which generates free radicals directly into the extracellular space (Babior, 1978).

Due to the very high degree of correlation between two key factors associated with blackspot bruising and oxidative burst, this suggests potential targets for future development of diagnostic systems for prediciting levels of blackspot susceptibility.

The increasing availability of tetrazolium compounds with increasing sensitivity to superoxide radicals could permit an accurate and rapid diagnostic system to be developed for the prediction of blackspot susceptibility in the field. The suppression of blackspot would appear to be more difficult – antioxidants are an obvious first choice and have been used extensively in the control of fruit and vegetable browning for many years – (Friedman, 1997). However reducing the oxidative capacity of an individual tuber, and thus reducing the capacity for blackspot pigment synthesis, would have effects throughout the cellular metabolism and could ultimately affect a key factor in potato production, for example, in pathogen challenge – if the potato had a heavily reduced capacity to produce active oxygen species then it might have a very much suppressed capacity to initiate an oxidative burst against an invading pathogen. Thus, potatoes adjusted to have suppressed oxidative burst would likely be highly susceptible to many bacterial and fungal pathogens – a scenario which would not be welcomed by the farming community.

In summary a novel biochemical response to mechanical impact in potatoes has been identified. The involvement of active oxygen species, specifically superoxide, has been proposed and is suggested to be of paramount importance in the pigment production (blackspot susceptibility) of an individual potato variety.

5 The role of polyphenol oxidase and tyrosine in pigment formation

5.1 Introduction

The involvement of both polyphenol oxidase and tyrosine in the biochemical formation of melanin when potato tubers are impacted has been studied for many years. The basic metabolic pathway is summarised in sections 1.3.2 and 1.3.3 of the introduction. In summary, the generally accepted mechanism is upon mechanical impact a degree of cellular decompartmentalisation takes place in which the enzyme polyphenol oxidase is released from the plastids into the cytosol and comes into contact with its subtrates, tyrosine and molecular oxygen. The reaction between tyrosine and molecular oxygen initially yields DOPA, which auto-oxidises through several intermediates to DOPA-quinone. The DOPA-quinone then undergoes a further reaction with polyphenol oxidase to yield melanin which further auto-oxidises and polymerises to form melanin polymers, giving the characteristic dark coloured pigmentation associated with blackspot bruising.

The invitation to carry out an industrial placement as a small part of an investigation into polyphenol oxidase activity at Horticulture Research International at Wellesbourne, Warwickshire with Dr S Clifford in lettuce allowed some alternative polyphenol oxidase assays to be adapted for use in the potato system.

Several groups have reported usage of superoxide radicals in preference to molecular oxygen by polyphenol oxidase in animal systems, a series of experiments were developed to ascertain usage of superoxide radicals by this enzyme in the potato system.

It was also of interest to investigate what forms of tyrosine the enzyme might be capable of utilising in pigment formation. This aspect has occupied a sizeable proportion of the blackspot biochemical research in recent years. In this investigation the ability of polyphenol oxidase to utilise tyrosine-rich peptides was compared to with the reaction using free tyrosine.

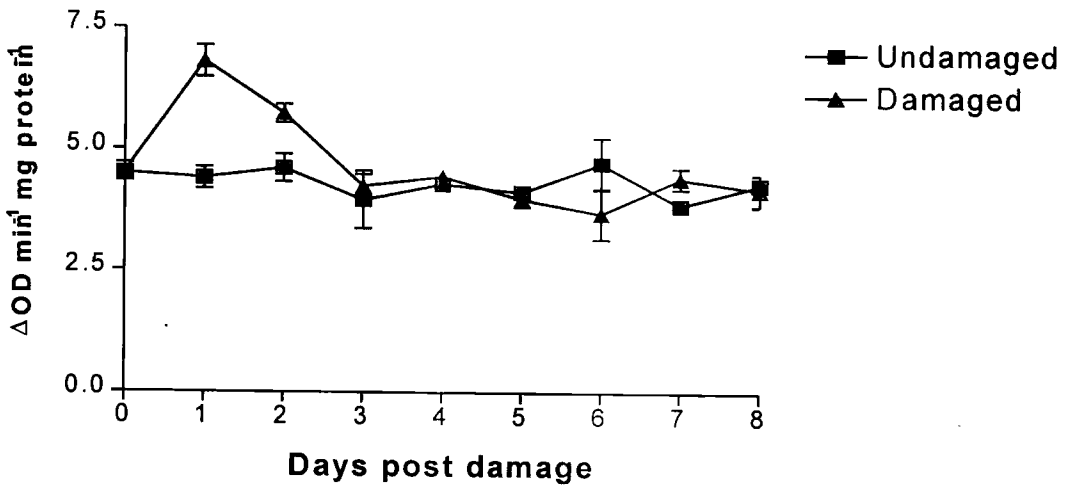
Finally the question as to the importance of tyrosine and polyphenol oxidase to the bruise pigment reaction was addressed by investigating what influence additions of excess substrate or enzyme were to the reaction.

5.2 Results

5.2.1 Level of PPO activity in potato over 8 days

An investigation was undertaken into potato polyphenol oxidase activity using a catechol oxidation assay (section 3.8.1) over a period of 8 days following mechanical impact. Tubers of Russet Burbank, selected because of its high susceptibility to blackspot bruise, were impacted, and, on a daily basis samples were taken and the catechol oxidation assay performed to assess PPO activity (figure 5.1). These data showed that in response to mechanical impact in potato there was a slightly elevated level of PPO reaching ~2 fold which then declined during the next 2 days before normal, background levels were re-established after 3 days. These results indicated that during the optimum pigment-producing period polyphenol oxidase acitivity was enhanced but normal levels returned within 3 days of impact. The basis of this activity enhancement is unknown.

Figure 5.1 : PPO activity in potato tubers following mechanical impact. Figure shows polyphenol oxidase activity in Russet Burbank potatoes over the 8 days following mechanical impact. Experiment performed as described in section 5.2.1. PPO assays carried out by oxidation of catechol (section 3.8.1). Experiment carried out in triplicate, error bars are SD of mean. The peak shown at 1 day post damage is not significant.



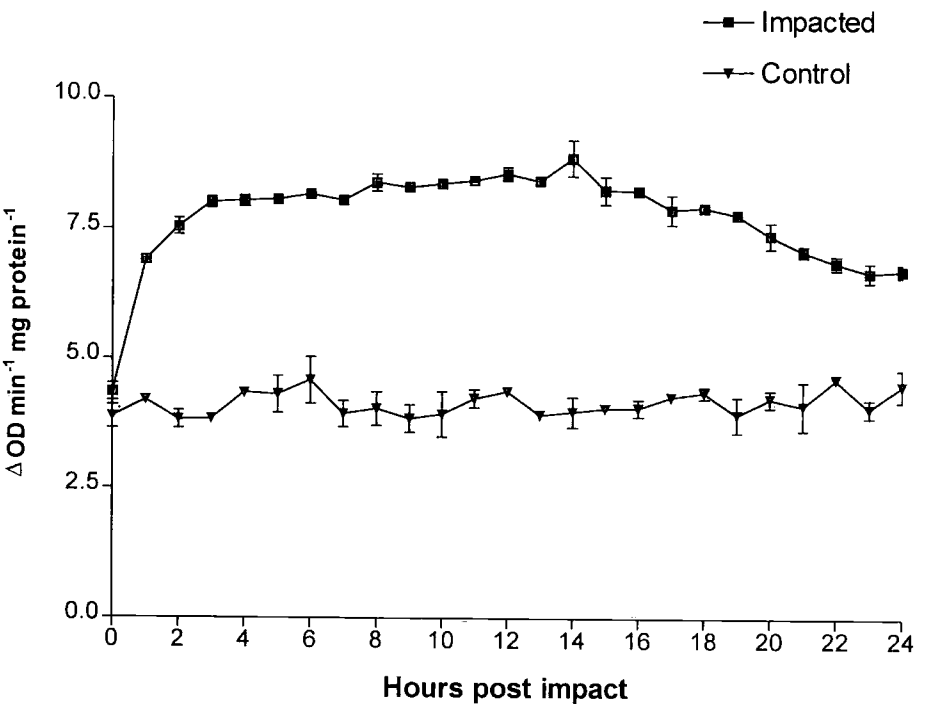
5.2.2 PPO activity in the tuber over 24 hours

After discovering elevated polyphenol oxidase activity in the 1-2 day post impact tuber samples a more detailed study was undertaken to analyse the changing activity of the enzyme over 24 hours. Russet Burbank tubers (again selected because of high blackspot susceptibility) were impacted and hourly samples extracted and assayed for polyphenol oxidase measured by the oxidation of catechol (figure 5.2). The results shown confirm that in the first 1-2 hours after mechanical impact there was a rapid increase in polyphenol oxidase activity. Subsequently up to 15 hours the polyphenol oxidase activity increased very gradually, followed during the period 15-24 hours by a decline in activity towards pre-impact levels. This profile of enhanced activity of polyphenol oxidase corresponds well with the time course of pigment development in blackspot bruise susceptible tubers.

5.2.3 The effect of active oxygen species in pigment formation

The results from section 4 demonstrated a rapid release of active oxygen species occurred immediately after mechanical impact in potatoes. The time course indicated an initial peak at 1-2 hours post impact. A possible explanation for the increasing PPO activity in the 1-2 hour post impact period was potentially through involvement of active oxygen species, as has been proposed in some animal systems. To investigate whether active oxygen species could influence polyphenol oxidase activity, an assay was established in which impacted Russet Burbank tuber sections were excised at hourly intervals post-impact and placed in a synthetic superoxide generating system (ascorbate-ferric chloride – section 3.5.1) for 15 minutes. This exposed the cells to a burst of radicals. The tissue was then extracted and assayed for PPO (using catechol oxidation – section 3.8.1) was then carried out and results compared to a sample of

Figure 5.2 : PPO activity in impacted Russet Burbank tubers. Polyphenol oxidase activity in Russet Burbank tubers assayed hourly over 24 hours following mechanical impact. PPO was assayed by oxidation of catechol as described in section 3.8.1. Each time point was carried out in triplicate, error bars are SD of mean.

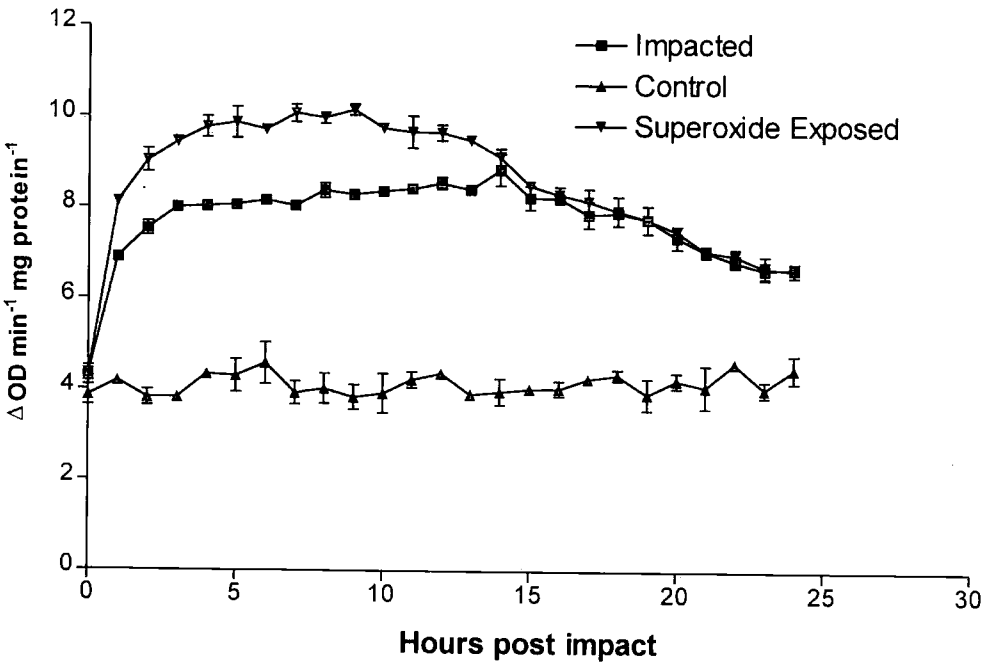


impacted tissue from the same tuber placed in a control (no ascorbate) solution (samples from 5.2.2) (figure 5.3). Over the range of time points from 0-14 hours after impact there was significant enhancement of polyphenol oxidase activity as a consequence of exposure to superoxide radicals. This was additional to the endogenous enhancement noted in the previous experiment. The highest enhancement was in the first 14 hours post-impact. After 14 hours, in the period where PPO activity was decreasing, the additional superoxide was unable to enhance enzyme activity, indicating that the PPO appeared to only be sensitive to superoxide enhancement in the first 14 hours after impact. After the 14 hours post impact time point no enhancement of PPO activity could be detected in response to exposure to superoxide, this time point corresponds approximately to the time at which active pigment synthesis ceases to take place (maximum pigment formation occurring after 15-24 hours at 27°C). Possible explanations for this behaviour are discussed in section 5.3.

5.2.4 Source of tyrosine for melanin synthesis in blackspot.

After tyrosine was established by several groups as having a high degree of correlation with blackspot bruise susceptibility, attention turned to the partitioning of the tyrosine pool between peptide-bound and free tyrosine and whether the percentage of tyrosine entering protein synthesis reduced after mechanical impact, allowing a greater quantity of substrate (free tyrosine) to be directed into for melanin synthesis. The question also arose as to whether tyrosine incorporated into protein could participate in the reactions. In order to investigate this, two synthetic tyrosine-rich peptides were obtained (Sigma, UK) and the degree of PPO-catalysed melanin synthesis measured and compared to that produced using free tyrosine as the substrate. The two peptides contained, in addition to tyrosine, glutamate and alanine in the following ratios : glu:ala:tyr 1:1:1

Figure 5.3: PPO activity in Russet Burbank after exposure to superoxide radicals. Figure shows enhancement of activity of PPO by exposure to superoxide radicals for 15 minutes prior to assay of PPO activity by catechol oxidation assay (section 3.8.1). The impacted data shown is that presented in figure 5.2, all time points were carried out in triplicate, error bars are SD of mean.



(GAT111), glu:ala:tyr 6:3:1 (GAT631), additionally a control peptide containing only glutamate and alanine was used: glu:ala 6:4 (GA64). The experiments were conducted using mushroom tyrosinase. Melanin produced from PPO from a wide variety of animal and plant species is widely held to be identical (Vaughn *et al.*, 1988), thus, in the absence of any structural data of PPO's we assume that the mechanism of action between mushroom tyrosinase and potato PPO is highly similar if not identical. Mushroom tyrosinase is therefore a convenient paradigm for PPO activities.

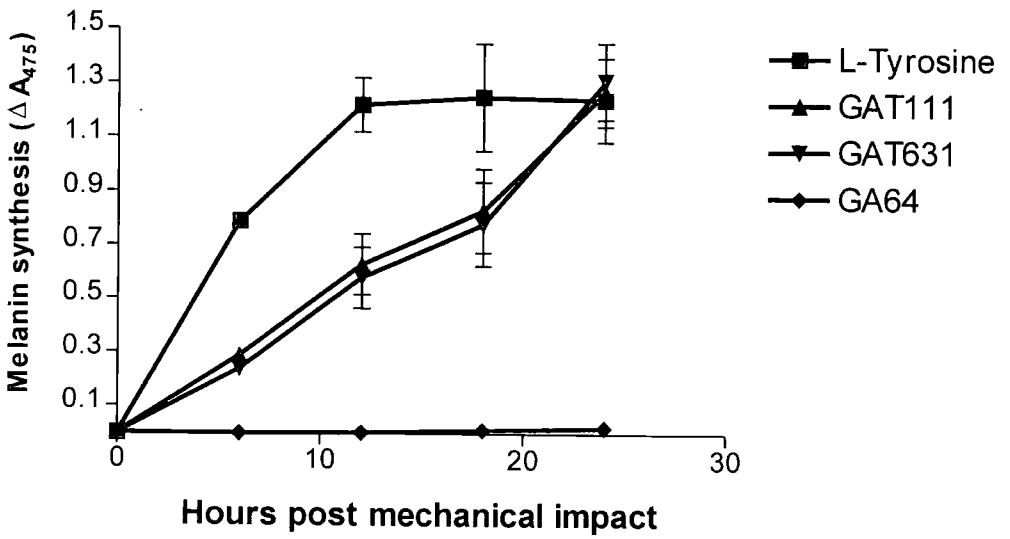
Table 5.1 outlines the maximal level of melanin achieved when each of the four substrates was reacted with mushroom tyrosinase (polyphenol oxidase). It indicates that over a 24 hour period less than 5% difference in overall pigment level was noted, whether the tyrosine substrate originated in a peptide (GAT111, GAT631), or as free amino-acid. This demonstrates that the PPO can utilise free or protein bound tyrosine without affecting the final yield of melanin, suggesting that the experiments carried out into the percentage of tyrosine entering protein synthesis are largely superfluous as this ratio has little or no importance in the potato tuber.

The rate of reaction might also be influenced by the source of tyrosine, kinetic results of melanin production by each of the peptides and free tyrosine (figure 5.4) suggest that although melanin yield is almost identical for each source of tyrosine after 24 hours, a level of variation exists in the rates of melanin production. Free tyrosine clearly shows in this assay it has the highest rate, maximal melanin production being achieved in 12 hours. The peptide bound tyrosine takes somewhat longer to achieve the maximal level, 24 hours in the case of GAT111 and GAT631. This suggests that although the source of tyrosine is largely inconsequential when considering overall production of

Table 5.1 : Melanin synthesis using various tyrosine substrates. Table shows maximum quantity of melanin synthesis after 24 hours, produced when PPO utilised free tyrosine, and tyrosine bound in two peptides. Melanin quantity assessed by absorbance at 475nm. Tyrosine peptides were GAT111 and GAT631 (glutamate : alanine : tyrosine ratio of 1:1:1 and 6:3:1 respectively). A control peptide GA64 (glutamate : alanine 6:4) also used. Assay was carried out as described in section 3.8.2 using 100μg/ml GAT631, 30μg/ml GAT111, 50μg/ml GA64, or 10μg/ml L-tyrosine and 25mg/ml mushroom tyrosinase (PPO).

Source of tyrosine	Melanin synthesis (absorbance units 475nm)	Control (no PPO) melanin synthesis (absorbance units 475nm)
L-Tyrosine	1.24 ± 0.11	0.08
GAT111	1.27 ± 0.09	0.05
GAT631	1.31 ± 0.10	0.01
GA64	0.02 ± 0.01	0.03

Figure 5.4 : Time course of melanin production using several tyrosine sources. Figure shows rate of melanin synthesis by mushroom PPO with various sources of tyrosine. GAT111 and GAT631 are synthetic peptides containing tyrosine (glutamate : alanine : tyrosine 1:1:1 and 6:3:1 respectively). Experiment carried out as described in section 5.2.4 in triplicate, error bars are SD of mean.



melanin, the rapidity of production is reliant upon availability of free tyrosine. GA64 showed no pigment producing capacity in this system confirming that all melanin originated from tyrosine residues in the two peptides. No experiments were performed to detect differences in the structure and size of the melanin products.

5.2.5 Limiting factors in the melanin synthesis pathway

Although tyrosine has been largely accepted as a key limiting factor in blackspot bruise, this has not been wholly agreed by all of the scientific community. To investigate whether it could be demonstrated that tyrosine was the limiting factor a straightforward experiment was established in which excess PPO was mixed with cell free extracts of tuber tissues containing cellular levels of tyrosine, and excess levels of tyrosine were mixed with cellular levels of PPO. For these purposes the figure referred to by Dean 1992 was assumed to be the mean cellular level of tyrosine (1.18μmol/mg) and that referred to by Friedman (1995) for the cellular level of PPO (3.72U/g). Excessive levels of either substrate or enzyme were taken to be 50 times the cellular level as this exceeds the cellular level of tyrosine or PPO at any time.

Figure 5.5 shows that when an excess of tyrosine was added to a Russet Burbank cell free extract it had no effect on pigment production over that of addition of mean cellular levels of tyrosine. Conversely figure 5.6 indicates that addition of excess PPO to the Russet Burbank tissue preparation caused a period of more rapid pigment production than that seen with mean cellular levels of PPO. Taken together these two figures suggest that contrary to the more popularly held concept that tyrosine acts as the limiting factor, it appears that PPO actually acts in this capacity. These findings are largely supported by recent work (Coetzer *et al*, 2001) who found in transgenic lines of

Figure 5.5 : Effect of adding excess tyrosine to cellular levels of PPO on melanin accumulation. Figure demonstrates the addition of excess (59μmol/mg) of free tyrosine to 3.72U/g PPO. Experiment was carried out as described in section 5.2.5 in triplicate, error bars are SD of mean.

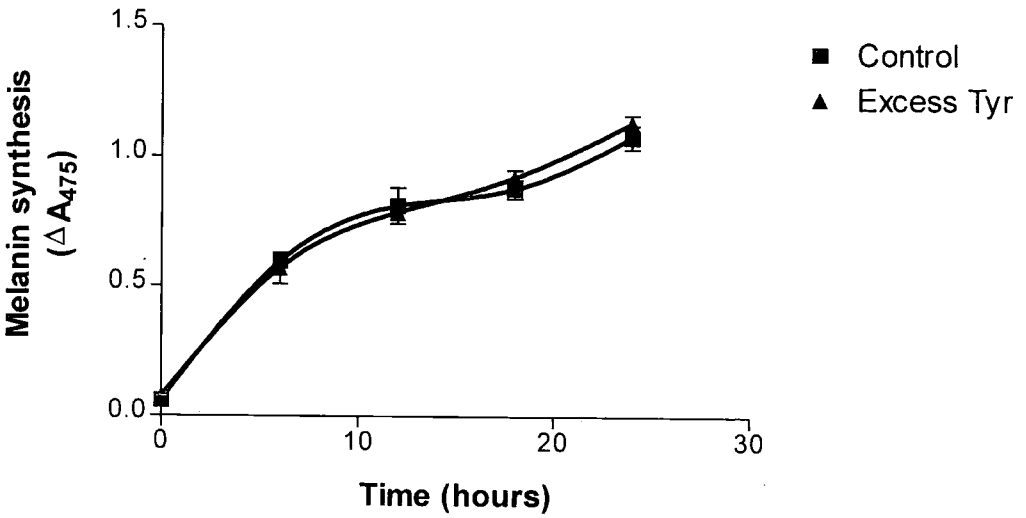
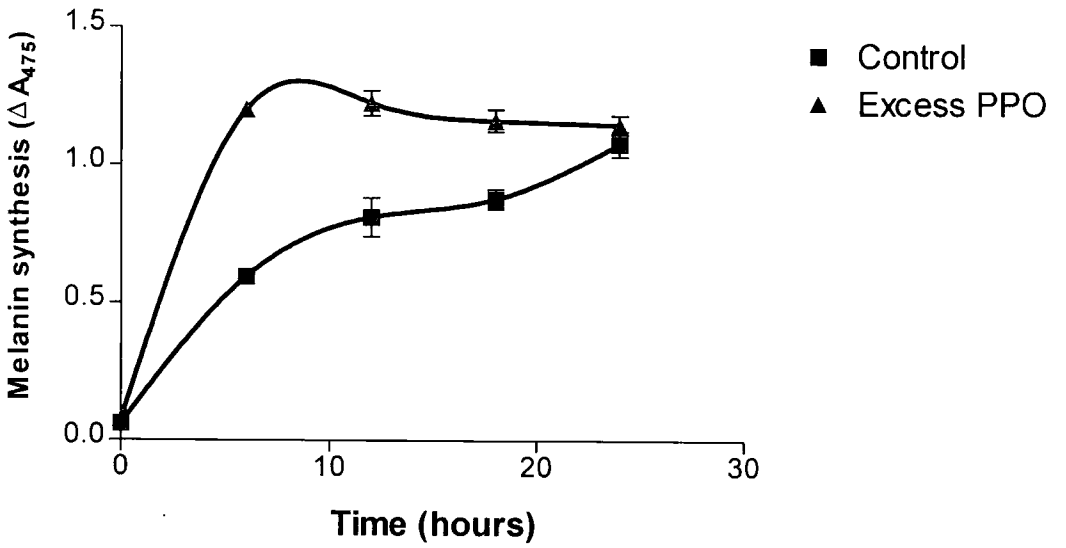


Figure 5.6 : Effect of adding excess PPO to cellular levels of tyrosine on melanin accumulation. Figure demonstrates the effect of addition of excess (186U/g) PPO to 1.18μmol/mg free tyrosine. Experiment was carried out as described in section 5.2.5 in triplicate, error bars are SD of mean.



anti-sense PPO the 5 of 28 lines which demonstrated reduced capacity for pigment production were those which showed reduced PPO activity. Furthermore when sense-tomato PPO was used there was almost no pigment production, thought to be due largely to co-suppression of the potato PPO. Note that none of these experiments takes into account the effects of spatial organisation of tissues and cells which might influence the PPO efficiency or reaction.

5.3 Discussion

PPO activity was found to be substantially increased in the first 14 hours after mechanical impact. Increased enzyme activity may be due to a number of factors e.g. enzyme concentration, cofactor concentration or activation of enzyme. This enhancement of PPO activity is not unexpected given the increased requirement for PPO immediately after mechanical impact for melanin synthesis. These findings are however contradictory to those of Laerke *et al* (2000) who noted no change in PPO of Maris Piper tubers over 32 hours. Interestingly in the period after 14 hours a decrease in PPO activity is observed, this time corresponds to the work of Partington *et al.* 1999 who claim that by 18 hours post-impact most cells in the impacted area became necrotic and this may be linked to overall reduction in PPO activity.

The enhancement of PPO activity by exposure to superoxide radicals is of significant interest and may in some way explain the contradiction in reports of PPO activities. As presented in section 4 Russet Burbank tubers undergo a substantial release of superoxide free radicals over the first 7 hours after mechanical impact. The PPO in this study was extracted immediately prior to PPO activity assay and hence, if the enzyme activity was already enhanced due to the presence of superoxide radicals, this could

explain the difference between these and the findings of Laerke *et al.* 2000 who used a more extended PPO extraction protocol before assaying enzyme activity. The enhancement of PPO activity by exposure to superoxide radicals poses two questions – how is the enhancement achieved and why does it take place?

One possible explanation as to how the enhancement takes place is that superoxide radicals have been demonstrated to modulate G-protein activity, as well as affecting other cell signalling components. In this way it could be possible for superoxide to modify signalling pathways controlling PPO activity. A possible mechanism for alteration of PPO activity could be that the area around the copper binding site in PPO is histidine rich, coincidentally histidine is a key target for oxidative modification (introduction of carbonyl groups), in this way alteration of structure around the metal binding site by exposure to free radicals may affect the activity of the enzyme. Alternatively, and this also addresses 'why' should the enhancement take place – superoxide radicals have been implicated as an alternative substrate to molecular oxygen for PPO in animal studies (Valverde *et al.*, 1996). Furthermore the use of superoxide radicals was preferential and more efficient by PPO in the system studied. In this system an excess of superoxide is present immediately after mechanical impact – if potato PPO also preferentially uses superoxide radicals over molecular oxygen then the presence of the superoxide potentially could enhance the efficiency of the enzyme, and consequently increase the rate of melanin synthesis. Valverde *et al.* 1996 also proposed that the use of superoxide radicals in melanin synthesis by PPO potentially confers a unique anti-oxidant protection mechanism to those cells rich in PPO.

As PPO appears to interact with superoxide radicals to enhance its activity it suggests potentially a more prominent role for this enzyme than has recently been proposed. The widely held tenet that tyrosine was the predominant factor determining blackspot pigment development appears to be slowly being dispelled. Work initiated by Bachem *et al.* (1994) using antisense PPO and its effects on blackspot sparked interest by industrial companies and other research groups into the enzyme. They noted that it was possible to reduce pigment production through the use of antisense PPO, but only if PPO was almost completely inhibited. More recently this has been modified by Coetzer *et al.* (2001) who undertook an anti-sense PPO study in which they found reduced levels of pigment production in 5 of 28 lines studied. These 5 lines all corresponded to lines of reduced PPO activity, though not, as previously suggested by Bachem, to completely inhibited PPO activity. Work by Stevens and Davelaar, (1997) also investigated the relationship of PPO to the bruising process. They noted that by separating the potential to produce blackspot pigments from the actual level of pigment produced after bruising they could only find a correlation between free tyrosine and the potential to produce blackspot pigments, and not with the actual pigment produced after bruising. From the work presented in section 5.2.5 it would appear that PPO is the major determining factor for the actual level of pigment produced in the tuber tissues after mechanical impact, albeit this was only tested in three of the varieties available. Mondy and Munshi (1993) noted a high degree of correlation between free tyrosine and level of enzymatic discolouration, however they also found that the most blackspot resistant cultivar had the highest levels of free tyrosine. They concluded that another factor, proposed to be propensity towards cellular decompartmentalisation, must also have a significant influence upon enzymatic discolouration.

Stevens *et al.*, (1998) investigated the structure of melanin pigments produced after mechanical impact and found a high percentage of pigment was proteinaceous in composition. This protein matrix incorporating various dark coloured phenolics was reported by the authors to be caused by random reactions occuring at the site of impact – developed from their suggestion that at the site of impact a complete collapse of cellular structures take place and a wholly random series of events occurs ultimately leading to dark coloured pigments being deposited. A more tightly controlled series of reactions is proposed here, based upon the high degree of correlation exerted over superoxide generation as described in section 4. If the processes are not completely random then it would seem likely that tyrosine residues within peptides are being utilised by PPO, as suggested by the work presented in section 5.2.4 using synthetic tyrosine containing peptides. The differing kinetic rates between usage of free tyrosine and peptide bound tyrosine is interesting – as both free and peptide bound tyrosine are present in the cytosol then free tyrosine would by utilised rapidly, quickly producing melanin, followed by a slower but more sustained melanin production as tyrosine bound into peptides was utilised. This could be the interpretation for figure 5.6 in which the first 5 hours shows a higher level of melanin production than the subsequent 20 hours.

6 The effect of metal ions in blackspot

6.1 Introduction

The effect of metal ions in influencing susceptibility of potato tubers to blackspot bruising has been known for more than 80 years and has formed the basis for the use of prescribed fertilizer regimes for growing potatoes to reduce blackspot bruising.

It was therefore interesting to test the effects of a number of metal ions directly on pigment synthesis and also to investigate whether any metal ion induced changes to blackspot sensitivity could be matched with changes in the oxidatively modified protein level, as measured by protein carbonyl group accumulation. An experimental system was thus established in which tubers were impacted and immediately sliced in half through the impacted site. After washing to remove cellular debris one half tuber was placed in a metal ion solution for 24 hours, the other half was immersed in deionised water as a control. Tuber halves were then subjected to bruise intensity assessments and secondary carbonyl group assays. A cuvette-based assay (section 3.9.1) was also developed to allow *in vitro* pigment development to be quantified. In this system tyrosine and PPO were mixed in an ion containing solution and pigment development monitored spectrophotometrically during the following 24 hours.

6.2 Results and Discussion

Eight metal ions (Ca^{2+} ; Cu^{2+} ; Fe^{3+} ; K^{+} ; Mg^{2+} ; Mn^{2+} ; Na^{+} ; Zn^{2+}) were selected for this study, based upon a variety of previous studies into the effects of metal ions upon blackspot bruising, and additionally upon those mineral ions required, albeit in trace quantities, for growth and development of plants. Each metal ion was tested for its

ability to affect bruise index ; pigment synthesis and carbonyl accumulation over a 24 hour time period.

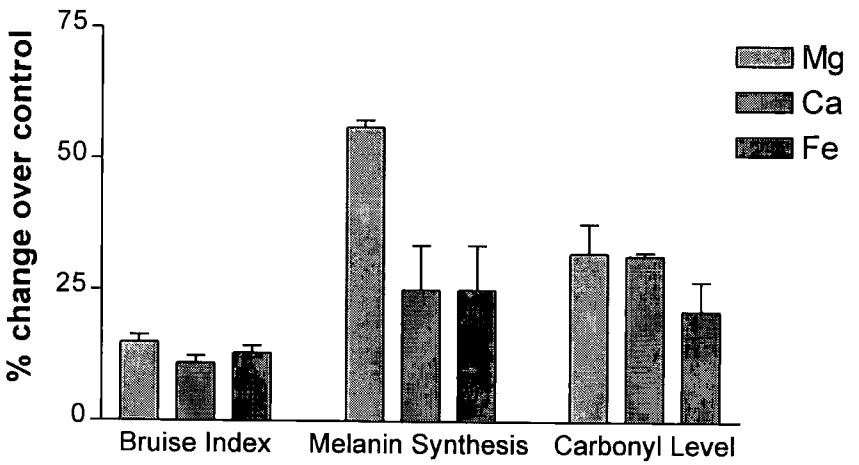
6.2.1 Magnesium, calcium and iron

Three metal ions (Mg ; Ca ; Fe) were shown to increase significantly pigment synthesis in Russet Burbank tubers when assayed by the *in vitro* system described in section 3.9.1. Concomitantly these metal ions were also shown to increase oxidative protein modification (as assayed by protein carbonyl levels) (figure 6.1).

Magnesium appeared to have the greatest effect, increasing the bruise index by 13%, pigment synthesis by 56% and carbonyl modification by 32%. The apparent discrepancy between increased pigment synthesis (of over 50%) compared to the slight increase in bruise index is largely due to the already highly susceptible nature of Russet Burbank potatoes to blackspot. Although the data suggest there is considerably more pigment synthesis taking place, the overall appearance of the bruise is still 'maximum intensity' – an analogy being painting with black paint, it is not possible to make a black object 'blacker'. Magnesium has not been reported as significant in blackspot bruising, however it has been positively correlated with the oxidative burst, showing the potential to induce an oxidative burst in differentiated HL-60 cells (Percival and Smith, 1994). As described in section 4.3, the ability to induce a superoxide burst can have a direct effect upon blackspot bruise index.

Calcium was also able to increase bruise index, albeit to a lesser degree than magnesium. A corresponding increase in secondary carbonyl levels and pigment production was also noted. Contradictory to these findings, calcium has been shown to

Figure 6.1 : Effect of magnesium, calcium and iron on blackspot. Figure demonstrates the positive effect Mg, Ca and Fe have on three blackspot related factors in Russet Burbank tubers. Experiments were carried out as described in section 3.9.1. Experiments carried out in triplicate, error bars are SD of mean.



have a negative effect upon blackspot in potatoes (Silva *et al.* 1991), however the wide range of cellular processes modulated by calcium transport e.g. cellular signalling would tend to suggest that excess of calcium in the extracellular medium might disrupt many biological pathways and mechanisms.

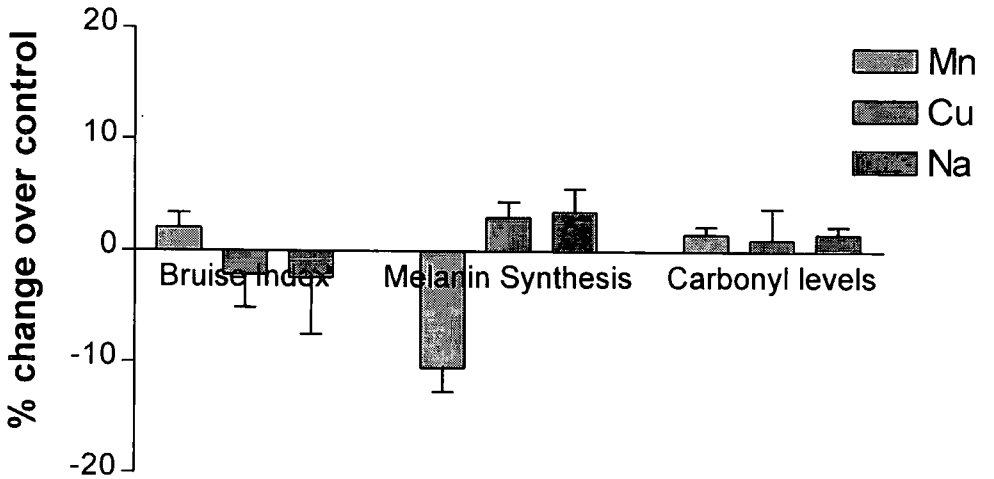
Iron also showed the ability to enhance bruise index, pigment production and carbonyl levels. This mineral ion has not been implicated in blackspot bruising agriculturally, however it does possess the ability to catalyse many oxidation reactions, generating superoxide radicals in the process. In this way the ion may be exerting influence over the blackspot pathway.

6.2.2 Manganese, copper and sodium

Three metal ions (manganese, copper and sodium) were all shown to have no significant effect on blackspot bruising (figure 6.2). None of the ions had any significant effect upon bruise index, pigment synthesis or secondary carbonyl accumulation.

No specific work connecting manganese to blackspot bruising in potatoes has been described however two studies in other systems may be of note - manganese has been demonstrated to have the ability to initiate a superoxide burst in HeLa cells in suspension culture (Percival and Smith, 1994), though the mechanism by which it does this is not known. In peas a negative correlation between manganese concentration and severity of blackspot has been demonstrated (Davidson and Ramsey, 2000). These two findings are at odds from a potato blackspot perspective, the ability to induce a

Figure 6.2 : Effect of manganese, copper and sodium on blackspot. Figure demonstrates the neutral effect Mn, Cu and Na have on three blackspot related factors in Russet Burbank tubers. Experiments were carried out as described in section 3.9.1. Experiments were carried out in triplicate, error bars are SD of mean.



superoxide burst, but also suppress blackspot may combine to cancel each other, potentially explaining the lack of effect of this mineral.

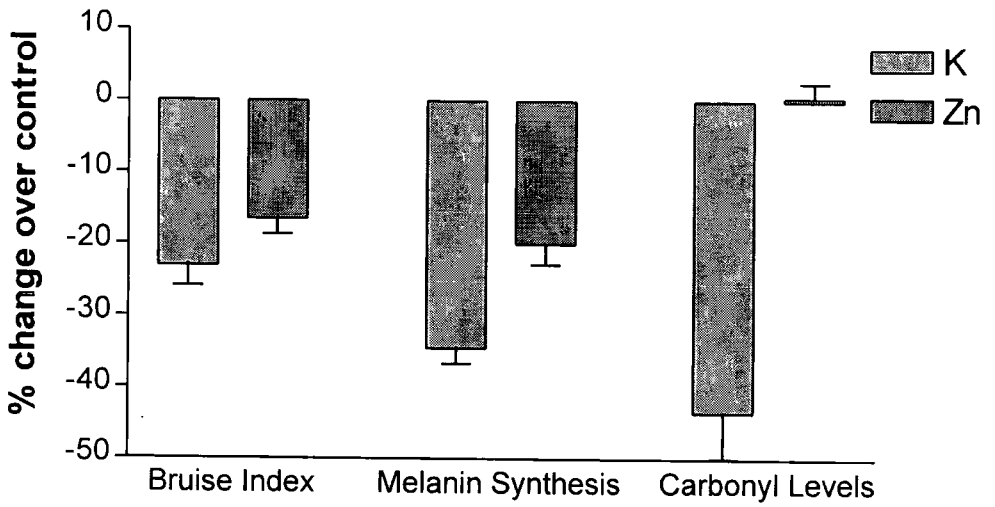
Copper is a co-factor for polyphenol oxidase and is essential for the reaction which produces melanin (Lerner, 1950). The first reaction catalysed by PPO (tyrosine to dihydroxyphenylalanine) has also been demonstrated to be accelerated by excess free copper (Kertesz, 1952). Additionally when potato plants were grown in a copper deficient media blackspot resistance increased (Mulder, 1956). Copper, has however been shown to be unable to stimulate an oxidative burst in suspension cells (Percival and Smith, 1994). These results here indicate the inability of exogenous copper to enhance blackspot bruising, however the ability to affect PPO directly within the timescale of this experiment is unlikely and would seem improbable.

Sodium has not been reported to have any effect on either blackspot or oxidative burst and therefore the inability to affect bruise index, pigment synthesis or carbonyl accumulation may be taken as confirmatory that this mineral has no effect on this system.

6.2.3 Potassium and Zinc

Both potassium and zinc were shown to have a large negative overall effect upon blackspot bruise – both minerals were able to reduce bruise index and pigment synthesis whereas potassium also reduced secondary carbonyl accumulation (figure 6.3).

Figure 6.3 : Effect of potassium and zinc on blackspot. Figure demonstrates the negative effect K and Zn have on three blackspot related factors in Russet Burbank tubers. Experiments were carried out as described in section 3.9.1 and were in triplicate, error bars are SD of mean.



The effect of potassium on blackspot in the field is a well established observation (Mondy *et al.*, 1967; Mulder, 1949; Mulder, 1956) and is the principal ionic fertilizer used on potato crops to modify blackspot susceptibility. The mode by which potassium reduces susceptibility to blackspot is not fully comprehended and the last relevant piece of work was that of Mondy *et al.*, (1967) who stated that potassium exerted its effect by decreasing levels of tyrosine though no molecular evidence for this statement was presented. The finding that potassium also significantly decreases the level of carbonyl modification may suggest a more complex explanation for the mode of action of this mineral suggesting a possible influence of potassium on the mechanisms associated with the oxidative burst e.g. ion transport.

Zinc, interestingly, was also able to reduce pigment synthesis and bruise index without apparently affecting secondary carbonyl levels. Like potassium, zinc has also been shown to have a negative effect upon blackspot bruising, and the application of zinc based fertilizers has been shown to increase resistance to blackspot. Due to severe effects upon plant growth zinc fertilizers are not now used commercially (Mondy and Chaudra, 1981). Once again the mode of action of zinc is not understood, again the most recent work (Mondy and Chaudra, 1981) suggests that the mineral works by reducing the level of tyrosine in the potato, though how zinc, or potassium, achieve this reduction is not explained. The fact that zinc does not affect secondary carbonyl levels suggests that zinc and potassium affect blackspot bruising by two different mechanisms.

6.2.4 Brass and steel

These experiments were set up as a consequence of some interesting observations made early in the programme in which prolonged contact between the metal corers used to excise tissue samples from tubers showed a striking enhancement of the blackspot bruise phenomenon. Brass and steel corers were used to remove mechanically impacted cores from tissues of Russet Burbank tubers (highly susceptible to blackspot bruise). The cores were incubated in the corer for 24 hours at 27°C. After this period the cores were removed and the bruise index calculated (section 4.1) and an assessment of carbonyl accumulation made (section 4.2.5) (figure 6.4). The results showed that the brass corer yielded a core which scored a maximum of 10 out of 10 for bruise index, with over 50% of the core having turned much darker than in normal bruising. There was also a corresponding increase in protein carbonyl level observed. Steel had no additional effect upon bruise index or carbonyl accumulation when compared to figures calculated for normal bruising in this variety.

Gross physical examination of the brass core showed very extensive intense discolouration, whereas the core taken from the steel corer had only a normal level of bruise pigment development (figure 6.5). The core taken from the brass corer in addition to having turned black in excess of 50% of the core, the remaining non-black parts had turned bright-blue, presumably due to an effect of copper ions.

The effect of metallic brass upon blackspot bruise was somewhat unexpected. Results from section 6.2.2 suggested copper had no significant effect upon blackspot or the enzyme itself. This was despite the previously described role of this mineral as the metal cofactor for polyphenol oxidase and the observation that non-black parts of the

Figure 6.4 : Effect of brass and steel on bruise index and carbonyl level. Figure shows effect of coring with brass or steel corers upon two factors associated with blackspot bruise. Experiments were carried out as described in section 6.2.4 and were in triplicate, error bars are SD of mean.

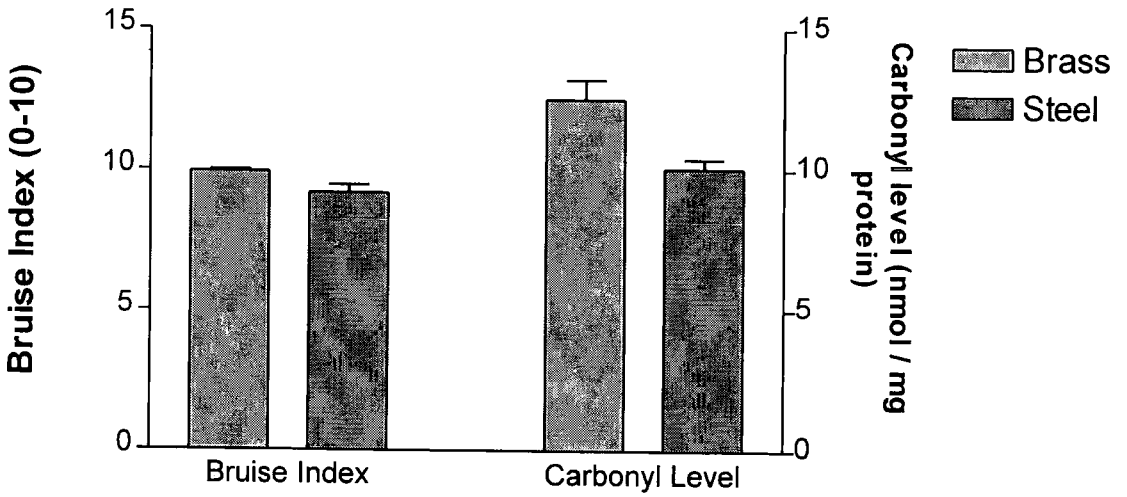
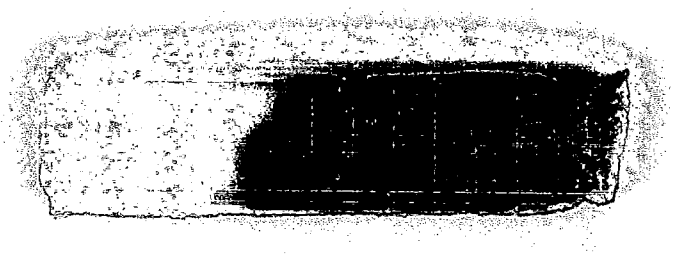


Figure 6.5 : Exposure of impacted tuber core to brass corer. Figure shows transverse section of tuber left in brass corer overnight.



tuber turned bright blue (a colour particularly associated with solutions of copper ions Cu^{2+}) would suggest that copper ions were responsible for this effect. Apparently when potato cores were left within the corer the copper ions from the metallic brass were more able to interact with the blackspot pathway than when tuber tissue was immersed directly in a solution rich in copper ions. This effect may be elucidated by work by Thygesen *et al.*, 1995 who, through work on the gene structure of PPO has identified a histidine-rich area conserved between PPO sequences from different families. The histidine rich region has been proposed to be the area where copper is bound into the PPO structure. Coincidentally histidine residues are also a primary target for modification by superoxide radicals, introducing secondary carbonyl side groups. In this way it could be envisaged that superoxide radicals present in the impacted core could be altering the secondary structure of the histidine rich regions possibly facilitating the binding of further copper ions, leading to an enhancement of PPO activity.

6.3 Summary

The effect of eight mineral ions on blackspot bruising has been presented. The eight mineral ions have been shown to have differing effects upon blackspot, ranging from enhancement of pigment synthesis to reduction of bruise index. A variety of factors have been proposed to cause the effects of the metal ions, some probably affect blackspot components directly – tyrosine levels and PPO activity, whereas others may act through modulation of components associated with the oxidative burst. Overall this is an area of blackspot research which has received little attention recently, however the interaction of mineral ions with the mechanically-induced oxidative burst suggests

further studies are required. A fuller study would also be able to investigate synergistic effects between combinations of these, and other, metal ions.

7. Multiple impact studies

7.1 Introduction

Sabba and Dean (1994) together with work presented in section 4.2.9 identified a variation in bruise susceptibility across the different regions of the potato tuber. For example the bud end, shows a substantially reduced susceptibility to blackspot bruise. The work in section 4.2.9 further suggests that a reduced oxidative capacity exists across the tuber, again the bud end have a substantially lower level of superoxide generation compared to the stolon end in response to identical mechanical impacts.

A collaborative study carried out with Mr S Patterson (3^{rd} year project student) suggested that when tubers of cv. Russet Burbank were mechanically impacted twice in different positions, <10 seconds apart, the second impact point reproducibly developed a blackspot bruise of lower bruise index (section 4.1) than the first point of impact. In this way by impacting first at the bud end (reduced blackspot susceptibility), followed 10 seconds later by impact at the stolon end (increased blackspot susceptibility) the relative susceptibility of each end could be reversed, the bud end developing the bruise with the highest bruise index.

This observation was subsequently studied by quantifying the mean bruise indices over a larger sample size and by assaying tubers of five varieties for accumulation of secondary carbonyl structures, indicative of exposure to active oxygen species.

7.2 Results

7.2.1 The effect of multiple impacts upon blackspot bruise susceptibility

Tubers of Russet Burbank were selected (because of its high blackspot susceptibility) and subjected to the multiple impact method (section 3.2), ensuring that secondary impacts were not diametrically opposite the initial impact which could lead to erroneous results. Half of the tubers assayed were bruised initially at the stolon end followed 10 seconds later by a secondary impact at the bud end. The other 50% were bruised initially at the bud end followed 10 seconds later by secondary impact at the stolon end.

Samples from both impact points, and a control (non-impacted) point, were assayed for superoxide production hourly over seven hours, by utilising the tetrazolium XTT based assay (section 3.5.5). Mean values for the primary impact site values (regardless of whether this was stolon or bud end bruising) and mean values for secondary impact site values were calculated and plotted (figure 7.1). This clearly showed that although the primary and secondary site of impact both followed the typical profile of superoxide generation (in two phases – section 4.3), in all cases the secondary impact sites were producing up to 40% less superoxide when compared to the primary sites. This confirmed the differential response of primary and secondary impact sites, and also demonstrated that this effect could negate the effect of position within the tuber, primary bud end bruises showing higher superoxide generation compared to secondary stolon end bruises.

A more detailed study was undertaken involving five varieties (representing the range of susceptibilities to blackspot). Each tuber was again subjected to two impacts, 10

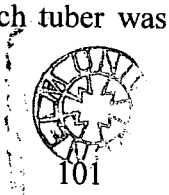
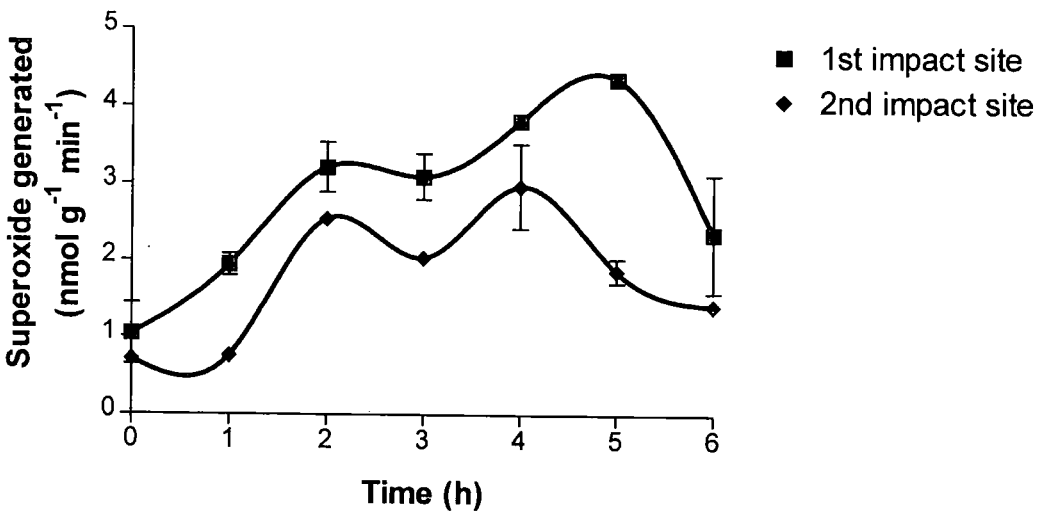


Figure 7.1 : Superoxide generation in Russet Burbank tubers after two impacts.

Figure shows effect of impacting a tuber twice on superoxide radical generation. Tubers were impacted ten seconds apart at opposite ends and assayed for superoxide generation as described in section 7.2.1. Experiment was carried out in triplicate, error bars are SD of mean. Impact sites were 50% bud end and 50% stolon to negate any effects conveyed by position on tuber.



seconds apart, 50% bruised at the bud end first, the other 50% at the stolon end. For each variety the bruise index and secondary carbonyl accumulation were assessed (table 7.1). In each variety tested the secondary point of impact had lower bruise index and lower secondary carbonyl accumulation compared to the first impact site. The differential susceptibility of the opposite ends of the tuber was retained when primary impact sites were compared and when secondary impact sites were compared, however the relative susceptibility to blackspot conferred by position in the tuber was not obvious when primary and secondary impact sites were compared to each other. In addition varietal effects on susceptibility were noted with Russet Burbank showing the highest bruise index and secondary carbonyl level through to Maris Piper which exhibited the lowest overall levels of bruise index and carbonyl accumulation.

7.3 Discussion

The reduction in blackspot bruising at secondary impact sites following a primary impact is an interesting observation, not least because tubers *are* likely to be impacted many times between harvesting and processing.

The immediate conclusion from this work is that following an initial impact tubers are desensitised to further impacts. The parallel reduction in superoxide producing ability also supports the hypothesis that active oxygen species generation is intimately linked with blackspot bruising in potatoes but also suggests that the superoxide generation system may actually determine the degree of response in terms of bruise pigment.

Exactly why such a reduction in bruise intensity and the associated factors should be observed is not altogether clear. The reduction in secondary bruising capability is

Table 7.1 : Bruise indices for tubers impacted twice. Table showing the effect on bruise index and carbonyl accumulation of impacting tubers twice. Tubers were either impacted at the bud or stolon end to negate any effects caused by position. Table a shows the effect upon bruise index (scale 0-10, where 10 represents the highest susceptibility to blackspot bruise). Table b shows carbonyl accumulation at the bruise impact site (nmol carbonyl / mg protein). Experiment carried out as described in section 7.2.1 in triplicate.

Table a

Variety	1st Stolon	2nd Bud	1st Bud	2nd Stolon
Russet Burbank	9.3 ± 0.2	3.7 ± 0.7	5.2 ± 0.6	3.4 ± 1.0
Saturna	7.7 ± 0.6	2.5 ± 0.3	4.2 ± 0.8	2.2 ± 0.3
Cara	5.0 ± 0.5	1.2 ± 0.1	3.0 ± 0.4	1.1 ± 0.2
King Edward	2.6 ± 0.2	1.0 ± 0.0	1.4 ± 0.2	0.8 ± 0.0
Maris Piper	1.0 ± 0.1	0.9 ± 0.0	1.0 ± 0.0	0.7 ± 0.2

Table b

Variety	1st Stolon	2nd Bud	1st Bud	2nd Stolon
Russet Burbank	10.4 ± 0.9	6.3 ± 1.1	8.5 ± 0.9	3.9 ± 1.1
Saturna	9.8 ± 1.2	5.7 ± 0.6	7.7 ± 1.1	2.9 ± 1.3
Cara	6.3 ± 0.8	3.5 ± 0.9	6.0 ± 0.4	2.6 ± 0.9
King Edward	5.0 ± 0.8	2.8 ± 1.0	3.5 ± 1.4	1.4 ± 0.8
Maris Piper	2.9 ± 1.0	1.2 ± 0.5	1.8 ± 0.3	0.9 ± 0.3

established extremely quickly, at most a few seconds of the initial impact the region of the secondary impact shows the reduced level of blackspot damage. This was too rapid an effect for a signalling molecule to travel the length of the tuber, even if it was present in all cells. Thus more likely is a mechanism involving transmission of a shock wave leading to induction of secondary responses which influences oxidative burst and bruise production.

In this model the first impact leads to the usual array of impact responses leading to the blackspot bruise at this site. From this site a shock wave or pressure wave is transmitted throughout the tuber. This type of energy transmission has been shown to have secondary effects and is an area of significant research in a wide range of biological systems (Ju *et al.*, 1999; Hilson and Pickering, 1994). In the tuber the pressure wave transmission could have a transient ion flux which in turn may have desensitised the cells to a further shock wave, this may be the whole basis of varietal differences – cell wall structures differ between varieties therefore different transmission of pressure wave and different responses to ion flux viz. different oxidative burst and different levels of bruise intensity.

The pressure wave caused a level of ion channel desensitisation to mechanical impact, possibly working through ion flux changes and perturbation of ion transporters. This desensitisation causes secondary impacts to have a substantially reduced effect. If ion channel changes cause the desensitisation to mechanical impact (as has been implied in other energy transmission systems (Kushida *et al.*, 2001; Wu and Chen, 2000; Ghazi *et al.*, 1998) then it would not be unreasonable to suggest that due to the effect of ion channel flux as a probable initiator of G-protein cascades, of the type which activates

the NADPH-oxidase complex, the ion imbalance may not permit a full release of active oxygen species to be released at a secondary impact site.

DISCUSSION

8. Summary

The following highlighted results and conclusions are drawn from this piece of work:

- Potato tubers show considerable variation in susceptibility to blackspot bruise between varieties
- Impacted tubers show a high degree of carbonyl group accumulation in their proteins, indicative of exposure to active oxygen species
- Carbonyl levels are very highly correlated with the degree of susceptibility to blackspot
- Superoxide radicals are generated in tuber tissues within the first 7 hours after impact
- The generation of superoxide radicals follows a characteristic biphasic profile.
- Superoxide generation as a result of mechanical impact is likely through a transmembrane NADPH Oxidase
- Superoxide generation is highly correlated with susceptibility to blackspot bruise
- Superoxide is responsible for most of the effects of active oxygen species in this system, hydrogen peroxide plays little or no part.
- PPO activity increases rapidly in the first 2 hours after impact and progressively increases up to 14 hours before declining to normal activity over a further 10 hours.
- Superoxide radicals enhance the activity of PPO up to 14 hours after impact but have no effect after this time.
- PPO utilises free- and protein bound-tyrosine with equal ease, however melanin synthesis is faster with free tyrosine

- PPO, and not tyrosine, may be the limiting factor in pigment synthesis in Russet Burbank, alternatively factors associated with free radicals may be limiting.
- Copper ions enhance blackspot pigment formation substantially, whereas potassium and zinc reduce susceptibility
- Desensitisation to secondary impacts is established within a few seconds of impact and may be established by ion channel perturbations caused by transmission of a pressure wave through the cells.

The demonstration throughout this project that active oxygen species, particularly superoxide free radicals, play an integral part in the responses of potato tubers to mechanical impact is highly significant. Referring to the diversity of reactions with which free radicals have been implicated in a wide variety of systems, a number of novel potato responses can be postulated, and previously described responses modified to reflect the involvement of active oxygen species. By drawing on results presented in this work and on results published in the scientific literature a model of the biochemical responses to mechanical impact in potatoes is proposed.

The hypothetical (and speculative) model is presented in the form of a time-course of events leading to bruising responses as this best demonstrates the interaction of several reactions.

8.1 Pre-impact events

Prior to mechanical impact a number of factors influence the susceptibility of a particular tuber or particular variety to blackspot bruising. Mondy and Munshi, (1993) suggest that tuber maturity directly influences blackspot through alteration of tyrosine

levels, the substrate for the melanin synthesis reaction. Corsini *et al.*, 1992 suggest that tyrosine levels within tuber varieties confers a degree of blackspot susceptibility, however this is disputed by Mondy and Munshi, (1993) who note that the most bruise resistant variety they tested also had the highest level of tyrosine. Dean *et al.*, (1992) labelled a precursor of tyrosine and found that in susceptible varieties the level of free (as opposed to protein incorporated) tyrosine was crucial.

The interaction of cells at the point of mechanical impact has also been studied – Hudson, (1975) suggested that large intercellular spaces and large cells tended to confer susceptibility to blackspot – this makes sense since large cells might be more mechanically sensitive than small ones. Storage of tubers also increases blackspot susceptibility – probably due to increasing tuber maturity, which has been correlated with increased tyrosine levels (Nylund *et al.*, 1955).

Various chemical treatments can also affect blackspot susceptibility – ethylene in low concentrations has been proposed to reduce susceptibility (Timm *et al.*, 1976), whereas in high concentrations has been shown to increase tuber discolouration, possibly through an increase in respiration rate (Ophius *et al.*, 1958). The minerals potassium and zinc also reduce susceptibility and are proposed to effect this through reduction in tyrosine levels (Mondy *et al.*, 1967, Mondy and Chaudra, 1981).

As a consequence tubers already have a predisposition to blackspot susceptibility influenced by environmental and physiological factors.

8.2 Impact

At the point of impact in a bruise susceptible variety (e.g. Russet Burbank) an energy is imparted at a precise localised spot on the tuber surface, the energy of which is dispersed through a pressure wave which passes through the whole tuber. This impact initiates a series of reactions as well as a shock wave which is proposed to travel through the tuber (section 7). The shockwave may alter ion channel operation, as has been suggested in other systems (Ju *et al.*, 1999) and causes a desensitisation to further impacts, possibly through redistribution of ions. This is established within the first few seconds after impact.

At the same time in the vicinity of the impact, plastid membranes are ruptured and the contents, including the copper-based metalloenzyme polyphenol oxidase (PPO), are released into the cytosol (Corsini *et al.*, 1992). Although internal membranes are being disrupted, in the case of blackspot bruising cell wall fractures are not evident (Brook, 1996 though Croy *et al* (1998) suggest some distortion of cell walls are apparent.

Also occurring within the first few seconds of mechanical impact initiation of events leading to induction of a rapid and sustained release of superoxide free radicals (section 4). As a direct result of mechanical impact, depolarisation of ion channels may occur, for example potassium channels (Laerke *et al.*, 2000). The ion channel depolarisation initiates G-protein signalling cascades which in turn are proposed to activate a transmembrane NADPH-oxidase enzyme which directly generates superoxide radicals into the extracellular space. The living cells in the vicinity of the impact have now initiated a full stress response.

Within 2 hours of the impact exposure to superoxide radicals has caused the activity of polyphenol oxidase to increase above normal levels (section 5). Where possible superoxide is being utilised by PPO in preference to molecular oxygen because of increased overall efficiency with this substrate (Valverde, 1996), likewise free tyrosine is predominantly being utilised preferentially by PPO (section 5). Large quantities of reaction intermediates are now being formed and to the naked eye the site of impact has a reddish tinge, indicative of the accumulation of pre-melanin intermediates such as dihydroxyphenylalanine (DOPA). Proteins are now showing the characteristic signs of damage caused by exposure to active oxygen species and other free radicals – typically the introduction of carbonyl side groups is seen on susceptible amino acid residues, this may weaken the overall stability of the affected proteins, leaving them more open to denaturation and proteolysis (Beckman and Ames, 1998, Dean *et al.* 1997, Stadtman *et al.* 1992, Grune *et al.*, 1997).

8.3 2-4 hours post-impact

In the period 2-4 hours after impact several key changes are occurring to the biochemical pathways, the initial surge of activity caused by the shock of impact is beginning to subside. G-protein activation of the NADPH oxidase complex may reduce, and a typical reduction in superoxide generation is observed (section 4). However the initial 2-3 hour peak of superoxide production may have introduced oxidative scission of cell wall polysaccharides (Fry, 1998) generating many oligosaccharide fragments. Some of these fragments may act as self-elicitors in much the same way as pathogen-plant interactions and potentially interact with surface receptors on the plant cell (putative results post-project suggest this is indeed the case) – the self-elicitation then reactivates the signalling cascade and the NADPH oxidase

begins superoxide generation once again, giving the characteristic biphasic production of superoxide , seen in both pathogen systems (Lamb and Dixon, 1997) and in the potato mechanical system (section 4).

PPO also undergoes changes at the period 2 hours after impact. At this point superoxide production is reducing (section 4), increasing the necessity for more molecular oxygen to act as subtrate, also free tyrosine levels have diminished rapidly so tyrosine incorporated into proteins becomes the principal source of substrate – both molecular oxygen and protein bound tyrosine are used less efficiently by PPO (Kubo *et* al., 2000, section 5) and the activity of the enzyme consequentally ceases to increase exponentially, instead becoming a more linear increase (section 5).

Melanin deposition is now beginning to take place, predominantly around the edges of cell membranes, and cell walls. This compound, through the conjugated aromatic benzene ring functional group possesses the ability to scavenge free radicals, sequestering the radicals through the aromatic benzene ring and may act to protect the delicate lipids and proteins in the surfaces of cell membranes, as has been propsed by Jacobson and Hong, (1997).

8.4 4-14 hours post impact

In the period 4-14 hours after impact, melanin polymerisation and deposition continues, though due to the use of protein bound tyrosine the melanin has a high proteinaceous component (Stevens and Davelaar, 1997). Superoxide generation is largely completed by 7 hours after impact, the antioxidant melanin and scavenging enzyme superoxide dismutase effectively controlling any residual generation. PPO now uses only protein

bound tyrosine and molecular oxygen which causes pigment synthesis to plateau (section 5).

8.5 Effects post-14 hours after impact

After 14 hours the stress response is largely over. Melanin synthesis decreases as PPO activity falls. In tandem apoptosis, known to be initiated by active oxygen species (Jabs, 1999) has now taken over – proteins already rendered fragile by oxidative modification are degrading, most cells in the impact site show no response to vital staining and the area of the 'bruise' is now largely dead cell matter (Partington *et al*, 1999). Over the next few hours dehydration of the impact zone takes place and a hole may form, typical of many severe blackspot bruises.

This speculative model attempts to incorporate the findings from this thesis onto a framework of published work on oxidative responses to blackspot bruise with existing research in the field. However, due to the highly contradictory nature of some aspects of blackspot research the application of oxidative data to some areas is highly subjective. The model incorporates aspects of this work which strongly support the hypothesis stated earlier that 'synthesis of blackspot bruise pigments is quantitatively determined by the level of generation of active oxygen species'. Parts of the model are suggested from observations seen in other plant or animal systems and are largely unsupported by research in potatoes and thus could be open to change in due course, however several of the key stages have been demonstrated through earlier work and added through work carried out in this piece of work.

Through this work a clearly defined, novel role for superoxide free radicals in mechanical impact in potatoes is proposed. This work will hopefully promote studies into how oxidative stress can be used to modulate blackspot bruising. Finally I hope this project represents a valuable contribution to the field of potato blackspot bruising.

BIBLIOGRAPHY

9. References

Abdullah, M.P. (1998) Biochemical and molecular studies of some aspects of disease resistance in potato. *Ph.D. Thesis, University of Durham*

Abe, J.I., Okuda, M., Huang, Q.H., Yoshizumi, M. and Berk, B.C. (2000) Reactive oxygen species activate p90 ribosomal s6 kinase via *Fyn* and *Ras*. *J Biol Chem* **275** 1739-1748

Adams, S., Green, P., Claxton, R., Simcox, S., Williams, M.V., Walsh, K. and Leeuwenburgh, C. (2001) Reactive carbonyl formation by oxidative and non-oxidative pathways. *Frontiers Biosci* **6** a17-24

Adler, V., Yin, Z., Tew, K.D. and Ronai, Z. (1999) Role of redox potential and reactive oxygen species in stress signalling. *Oncogene* **18** 6104-6111

Aguirre, T., Matthijs, G., Robberecht, W., Tilkin, P. and Cassiman, J.J. (1999) Mutational analysis of the Cu/Zn superoxide dismutase gene in 23 familial and 69 sporadic cases of amyotrophic lateral sclerosis in Belgium. *Eur J Hum Genet* **7** 599-602

Aruoma, O.I., Murcia, A., Butler, J. and Halliwell, B. (1993) Evaluation of the antioxidant and prooxidant actions of gallic acid and its derivatives. *J Agric Food Chem* **41** 1880-1885

Asami, S., Manabe, H., Miyake, J. (1997) Cigarette smoking induces an increase in oxidative DNA damage, 8-hydroxydeoxyguanosine, in a central site of the human lung. *Carcinogenesis* **18** 1763-1766

Auh, C.K. and Murphy, T.M. (1995) Plasma-membrane redox enzyme is involved in the synthesis of O_2^- and H_2O_2 by *Phytophthora* elicitor-stimulated rose cells. *Plant Physiol* **107** 1241-1247

Babior, B.M. (1978) Oxygen-dependent microbial killing by phagocytes. *NEJM* **298** 659-668, 721-725

Babior, B.M., Kipnes, R.S. and Curnutte, J.T. (1973) Biological defence mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. *J Clin Invest* **52** 741-744

Bachem, C.W.B., Speckmann, G.J., van der Linde, P.C.G., Verheggen, F.T.M., Hunt, M.D., Steffens, J.C. and Zabeau, M. (1994) Antisense expression of polyphenol oxidase genes inhibits enzymatic browning in potato tubers. *Biotechnology* **12** 1101-1105

Baker, C.J. and Orlandi, E.W. (1995) Active oxygen in plant pathogenesis. *Ann Rev Phytopathol* **33** 299-321

Baker, C.J., Deahl, K., Domek, J. and Orlandi, E.W. (1998) Oxygen metabolism in plant/bacteria interactions: Effect of DPI on the pseudo-NAD(P)H oxidase activity of peroxidase. *Biochem Biophys Res Comm* **252** 461-464

Beckman, K.B. and Ames, B.N. (1998) The free radical theory of aging matures. *Physiol Rev* **78** 547-581

Behl, C., Davis, J.B., Lesley, R. and Schubert, D. (1994) Hydrogen peroxide mediates amyloid ß protein toxicity. *Cell* **77** 817-827

Beers, E.P. (1997) Programmed cell death during plant growth and development. *Cell Death Differ* **4** 649-661

Bergey, D.R., Hoi, G.A. and Ryan, C.A. (1996) Polypeptide signalling for plant defensive genes exhibits analogies to defense signalling in animals. *Proc Natl Acad Sci USA* **93** 12053-12058

Bestwick, C.S., Brown, I.R., Bennett, M.H.R. and Mansfield, J.W. (1997) Localization of hydrogen peroxide accumulation during the hypersensitive reaction of lettuce cells to *Pseudomonas syringae* pv. *phaseolica. Plant Cell* **9** 209-221

Birecki, M., Bizien, H.J. and Henderson, H.M. (1971) Effect of culture, storage and variety on polyphenol oxidase and peroxidase activities in potatoes. *Am Pot J* **48** 255-261.

Bolwell, G.P., Butt, V.S., Davies, D.R. and Zimmerlin, A. (1995) The origin of the oxidative burst in plants. *Free Radic Res* **23** 517-532

Bolwell, G.P. (1996) The origin of the oxidative burst in plants. *Biochem Soc Trans* **24** 438-442

Bolwell, G.P., Davies, D.R., Gerrish, C., Auh, C-K. and Murphy, T. (1998) Comparitive biochemistry of the oxidative burst produced by rose and French bean cells reveals two distinct mechanisms. *Plant Physiol* **116** 1379-1385

Bowler, C., Slooten, L., Vandenbranden, S., de Rycke, R., Botterman, J., Sybesma, C., van Montagu, M. and Inze, D. (1991) Manganese superoxide dismutase can reduce cellular damage mediated by oxygen radicals in transgenic plants. *EMBO J* **10** 1723-1732

Bradford, M.M. (1976) A rapid and sensitive method for quantification of microgram quantities of protein utilising the principle of protein-dye binding. *Anal Biochem* **72** 248-254

Bradley, D.J., Kjellbom, P. and Lamb, C.J. (1992) Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: A novel, rapid defense response. *Cell* **70** 21-30

Brimble, L.J.F. (1957) In *Intermediate Botany.* London : Macmillan

Brisson, L.F., Tenhaken, R. and Lamb, C. (1994) Function of oxidative cross-linking of cell-wall structural proteins in plant-disease resistance. *Plant Cell* **6** 1703-1712

Brook, R.C. (1996) Potato bruising: how and why emphasizing blackspot bruise. Michigan. *Running Water Publishing.*

Burcham, P.C. and Kuhan, Y.T. (1996) Diminished susceptibility to proteolysis after protein modification by the lipid peroxidation product malondialdehyde: Inhibitory role for crosslinked and non crosslinked adducted proteins. *Arch Biochem Biophys* **340** 331-337

Busciglio, J. and Yankner, B.A. (1995) Apoptosis and increased generation of reactive oxygen species in Down's syndrome neurons *in vitro*. *Nature* **378** 776-779

Bustamante, J., Bredeston, L., Malanga, G. and Mordoh, J. (1993) Role of melanin as a scavenger of active oxygen species. *Pigm Cell Res* **6** 348-353

Cary, J.W., Lax, A.R. and Flurkey, W.H. (1992) Cloning and characterization of cDNAs coding for *Vicia faba* polyphenol oxidase. *Plant Mol Biol* **20** 245-253

Chai, H.B. and Doke, N. (1987) Superoxide anion generation: A response to potato leaves to infection with *Phytophthora infestans*. *Physiol Biochem* **77** 645-649

Chandra, S., Martin, G.B. and Low, P.S. (1996) The Pto kinase mediates a signalling pathway leading to the oxidative burst in tomato. *Proc Natl Acad Sci USA* **93** 13393-13397

Chauffe, L., Windle, J.J. and Friedman, M. (1975) Electron spin resonance study of melanin treated with reducing agents. *Biophys J* **15** 565-571

Chen, Z., Silva, H. and Klessig, D.F. (1993) Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. *Science* **262** 1883-1886

Cimino, F., Esposito, F., Ammendola, R. and Russo, T. (1997) Gene regulation by reactive oxygen species. *Curr Top Cell Regul* **35** 123-148

Coetzer, C., Corsini, D., Love, S., Pavek, J. and Tumer, N. (2001) Control of enzymatic browning in potato (*Solanum tuberosum* L.) by sense and antisense RNA from tomato polyphenol oxidase. *J Agric Food Chem* **49** 652-657

Cohen, G. (1984) Oxy-radical toxicity in catecholamine neurons. *Neurotoxicology* **5** 77-82

Cole, C.S. (1975) Variation of dry matter between and within potato tubers. *Potato Res* **18** 28-37

Collen, J. and Pedersen, M. (1994) A stress-induced oxidative burst in *Eucheuma platycladum* (Rhodophyta). *Pysiol Plant* **92** 417-422

Constabel, C.P., Bergey, D.R. and Ryan, C.A. (1995) Systemin activates synthesis of wound-inducible tomato leaf polyphenol oxidase via the octadecanoid defense signalling pathway. *Proc Natl Acad Sci USA* **92** 407-411

Corsini, D.L., Pavek, J.J. and Dean, B. (1992) Differences in free and protein-bound tyrosine among potato genotypes and the relationship to internal blackspot resistance. *Am Pot J* **69** 423-435

Craft, C.C. (1966) Localization and activity of phenolase in the potato tuber. *Am Pot J* **43** 112-121

Crippa, P.R., Mazzini, A. and Salmelli, D. (1979) Oxidation of NADH by melanin: effect of UV light and copper ions. *Physiol Chem Phys* **11** 491-499

Croy, R.R.D., Baxter, R., Deakin, W., Edwards, R., Gatehouse, J.A., Gates, P., Harris, N., Hole, C., Johnson, S.M. and Raemaekers, R. (1998) Blackspot bruising in potatoes: structural and molecular approaches to the identification of factors associated with tuber bruising susceptibility. *Asp Appl Biol* **52** 207-214

Davidson, J.A. and Ramsey, M.D. (2000) Pea yield decline syndrome in South Australia: the role of diseases and the impact of agronomic practices. *Aus J Agric Res* **51** 347-354

Davies, G.R., Simmonds, N.J., Stevens, T.R.J., Grandison, A., Blake, D.R. and Rampton, D.S. (1992) Mucosal reactive oxygen metabolite production in duodenal ulcer disease. *Gut* **33** 1467-1472

Dean, B.B., Jackowiak, N. and Munck, S. (1992) Tyrosine synthesis in potato tuber tissue from blackspot-susceptible and resistant genotypes. *Potato Res* **35** 49-53

Dean, B.B., Jackowiak, N., Nagle, M., Pavek, J. and Corsini, D. (1993) Blackspot pigment development of resistant and susceptible *Solanum tuberosum* L. genotypes at harvest and during storage measured by three methods of evaluation. *Am Pot J* **70** 201-218

Dean, R.T., Fu, S., Stocker, R. and Davies, M.J. (1997) Biochemistry and pathology of radical-mediated protein oxidation. *Biochem J* **324** 1-18

Desikan, R., Hancock, J.T., Coffey, M.J. and Neill, S.J. (1996) Generation of active oxygen in elicited cells of *Arabidopsis thaliana* is mediated by a NADPH oxidase-like enzyme. *FEBS Lett* **382** 213-217

Dianzani, M.U. (1985) Lipid peroxidation in ethanol poisoning: a critical reconsideration. *Alcohol Alcohol* **20** 161-173

D'Ischia, M., Napolitano, A. and Prota, G. (1991) Peroxidase as an alternative to tyrosinase in the oxidative polymerisation of 5,6-dihydroxyindole to melanin. *Biochim Biophys Acta* **1073** 423-430

Dixon, T. (1992) Handbook of potato varieties. Potato Marketing Board (Oxford).

Doke, N. (1983) Involvement of superoxide anion generation in the hypersensitive response of potato tuber tissues to infection with an incompatible race of *Phytophthora infestans* and to the hyphal wall components. *Physiol Plant Pathol* **23** 345-357

Dry, I.B. and Robinson, S.P. (1994) Molecular cloning and characterisation of grape berry polyphenol oxidase. *Plant Mol Biol* **26** 495-502

Dudley, E.D. and Hotchkiss, J.H. (1989) Cysteine as an inhibitor of polyphenol oxidase. *J Food Biochem* **13** 65-75

Dwyer, S.C., Legendre, L., Low, P.S. and Leto, T.L. (1996) Plant and human neutrophil oxidative burst complexes contain immunologically related proteins. *Biochim Biophys Acta* **1289** 231-237

Ellis, R.E., Yuan, J. and Horwitz, H.R. (1991) Mechanisms and functions of cell death. *Ann Rev Cell Biol* 7 663-698

Espin, J.C., Veltman, R.H. and Wichers, H.J. (2000) The oxidation of L-ascorbic acid catalysed by pear tyrosinase.

Farkas, G.L. and Kiraly, Z. (1962) Role of phenolic compounds in the physiology of plant diseases and disease resistance. *Phytopath* **44** 8-150

Felix, C.C., Hyde, J.S., Sarna, T. and Sealy, R.C. (1978) Interactions of melanin with metal ions. Electron spin resonance evidence for chelate complexes of metal ions with free radicals. *J Am Chem Soc* **100** 3922-3926

Felton, G., Donato, K., Del Vecchio, R. and Duffey, S. (1989) Activation of plant foliar oxidases by insect feeding reduces nutritive quality of foliage for noctuid herbivores. *J Chem Ecol* **15** 2667-2694

Felton, G.W., Donato, K.J., Broadway, R.M. and Duffey, S.S. (1992) Impact of oxidized plant phenolics on the nutritional quality of dietary protein to a Noctuid herbivore, *Spodoptera exigua*. *J Insect Physiol* **38** 277-285

Flor, H.H. (1947) Inheritance of reaction to rust in flax. *J Agr Res* **74** 241-262

Flores, S.C., Marecki, J.C., Harper, K.P., Bose, S.K. and McCord, J.M. (1993) Tat protein of human immunodeficiency virus type 1 represses expression of manganese superoxide dismutase in HeLa cells. *Proc Natl Acad Sci USA* **90** 7632-7636

Friedman, M. (1991) Prevention of adverse effects of food browning. *Adv Exp Med Biol* **199** 171-215

Friedman, M. (1995) Improvement in the safety of foods by SH-containing amino acids and peptides. A review. *J Agric Food Chem* **42** 3-20

Friedman, M. (1996) Food browning and its prevention: an overview. *J Agric Food Chem* **44** 631-653

Friedman, M. (1997) Chemistry, biochemistry, and dietary role of potato polyphenols. A review. *J Agric Food Chem* **45** 1523-1540

Friedman, M. and Bautista, F.F. (1995) Inhibition of polyphenol oxidase by thiols in the absence and presence of potato tissue suspensions. *J Agric Food Chem* **43** 69-76

Friedman, M. and Molnar-Perl, I. (1990) Inhibition of food browning by sulfur amino acids. 1. Heated amino acid-glucose systems. *J Agric Food Chem* **38** 1642-1647

Friedman, M., Molnar-Perl, I. and Knighton, D. (1992) Browning prevention in fresh and dehydrated potatoes by SH-containing amino acids. *Food Addit Contam* **9** 499-503

Fry, S.C. (1998) Oxidative scission of plant cell wall polysaccharides by ascorbate-induced hydroxyl radicals. *Biochem J* **332** 443-453

Fukuda, H. (1997) Programmed cell death during vascular system formation. *Cell Death Differ* **4** 684-688

Ghazi, A., Berrier, C., Ajouz, B. and Besnard, M. (1998) Mechanosensitive ion channels and their mode of activation. *Biochim* **80** 357-362

Gilchrist, D.G. and Kosuge, T. (1980) Aromatic amino acid biosynthesis and its regulation. In: Miflin, B.J. Ed. *The biochemistry of plants* vol 5. New York , Academic Press.

Golan-Goldhirsh, A., Mozafar, A. and Oertli, J.J. (1994) Effect of ascorbic-acid on soybean seedlings grown on a medium containing a high-concentration of copper. *J Plant Nutr* **18** 1735-1741

Gorman, A., McGowan, A. and Cotter, T.G. (1997) Role of peroxide and superoxide anion during tumour cell apoptosis. *FEBS Lett* **404** 27-33

Green, T.R. and Ryan, C.A. (1972) Wound-induced proteinase inhibitor in plant leaves: a possible defense mechanism against insects. *Science* **175** 776-777

Greenlund, L.J., Deckwerth, T.L. and Johnson, E.M.Jr. (1995) Superoxide dismutase delays neuronal apoptosis: A role for reactive oxygen species in programmed neuronal death. *Neuron* **14** 303-315

Groom, Q.J., Torres, M.A., Fordham-Skelton, A.P., Hammond-Kosack, K.E., Robinson, N.J. and Jones, J.D.G. (1996) *RbohA*, a rice homologue of the mammalian *gp91phox* respiratory burst oxidase gene. *Plant J* **10** 515-522

Grune, T., Reinheckel, T. and Davies, K.J. (1997) Degradation of oxidized proteins in mammalian cells. *Faseb J* **11** 526-534

Hadfield, K.A. and Bennett, A.B. (1997) Programmed senescence of plant organs. *Cell Death Differ* **4** 662-670

Herrlich, P. and Bohmer, F.D. (2000) Redox regulation of signal transduction in mammalian cells. *Biochem Pharmacol* **59** 35-41

Hill, H.Z. (1992) The function of melanin or six blind people examine and elephant. *Bioessays* **14** 49-56

Horne, A.S. (1912) Bruise in potato. *J Roy Hort Soc* **38** 40-50

Hubel, C.A. (1999) Oxidative stress in the pathogenesis of preeclampsia. *Proc Soc Exp Biol Med* **222** 222-235

Hudson, D.E. (1975) The relationship of cell size, intercellular space, and specific gravity on bruise depth in potatoes. *Am Pot J* **52** 9-14

Hughes, J.C. (1980) Role of tuber properties in determining susceptibilities of potatoes to damage. *Ann App Biol* **96** 344-345

Hunt, M.D., Eannetta, N.T., Yu, H., Newman, S.M. and Steffens, J.C. (1993) cDNA cloning and expression of potato polyphenol oxidase. *Plant Mol Biol* **21** 59-68

Jabs, T. (1999) Reactive oxygen intermediates as mediators of programmed cell death in plants and animals. *Biochem Pharmacol* **57** 231-245

Jabs, T., Dietrich, R.A. and Dangl, J.L. (1996) Initiation of runaway cell death in an Arabidopsis mutant by extracellular superoxide. *Science* **273** 1853-1856

Jabs, T., Colling, C., Tschope, M., Hahlbrock, K. and Scheel, D. (1997) Elicitor-stimulated ion fluxes and O_2^- from the oxidative burst are essential components in triggering defense gene activation and phytoalexin synthesis in parsley. *Proc Natl Acad Sci USA* **94** 4800-4805

Jacobson, E.S. and Hong, J.D. (1997) Redox buffering by melanin and Fe(II) in *Cryptococcus neoformans. J Bacteriol* **179** 5340-5346

Jacobson, M.D. and Raff, M.C. (1995) Programmed cell death and Bcl-2 protection in very low oxygen. *Nature* **374** 814-816

Jensen, R.A. (1986) Tyrosine and phenylalanine biosynthesis relationship between alternate pathways, regulation, and subcellular localisation. *Rec Adv Enzymol* **20** 57-81

Jimbow, K., Fitzpatrick, T.B. and Quevedo, W.C.Jnr (1984) Formation, chemical composition and function of melanin pigments. In Matolsty, A.G. Ed. *Biology of the integument* vol 2. New York:Springer

Johns, T. and Keen, S. (1986) Taste evaluation of potato glycoalkaloids by the Aymara: a case study in human chemical ecology. *Human Ecol* **14** 437-452

Johnson, T.M., Yu, Z-X, Ferrans, V.J., Lowenstein, R.A. and Finkel, T. (1996) Reactive oxygen species are downstream mediators of p53-dependent apoptosis. *Proc Natl Acad Sci USA* **93** 11848-11852

Jones, G.H. (1994) Activation of ATP-GTP 3'-pyrophosphotransferase (guanosine pentaphosphate synthetase) in *Streptomyces antibioticus*. *J Bacteriol* **176** 1482-1487

Jones, A.M. and Dangl, J.L. (1996) Logjam at the Styx: programmed cell death in plants. *Trends Plant Sci* **1** 114-119

Kaaber, L. (1993) Glycoalkaloids, green discoloration and taste development during storage of some potato varieties, *Solanum tuberosum* L. *Norw J Agric Sci* **7** 221-229

Kerr, S., Brosnan, M.J., McIntyre, M., Reid, J.L., Dominiczak, A.F. and Hamilton, C.A. (1999) Superoxide anion production is increased in a model of genetic hypertension: role of the endothelium. *Hypertension* **33** 1353-1358

Kertesz, D. (1952) Tyrosinase and polyphenoloxidase. The role of metallic ions in melanogenesis. *Biochem Biophys Acta* **9** 170-179

Killick, R.J. and MacArthur, A.W. (1980) The relationship between bruising and specific gravity in some potato varieties. *Potato Res* **23** 457-461

Klann, E., Roberson, E.D., Knapp, L.T. and Sweatt, J.D. (1998) A role for superoxide in protein kinase C activation and induction of long-term potentiation. *J Biol Chem* **273** 4516-4522

Korytowski, W., Pilas, B. and Sarna, T. (1987) Photoinduced generation of hydrogen peroxide and hydroxyl radicals in melanins. *Photochem Photobiol* **45** 185-190

Kosuge, T. (1969) The role of phenolics host response to infection. *Ann Rev Phytopathol* 7 195-222

Kowalski, S.P., Plaisted, R.L. and Steffens, J.C. (1992) Immunodetection of polyphenol oxidase in glandular trichomes of *S.berthaultii, S.tuberosum* and their hybrids. *Am Pot J* **70** 185-199

Kubo, I., Kinst-Hori, I., Kubo, Y., Yamagiwa, Y., Kamikawa, T. and Haraguchi, H. (2000) Molecular design of antibrowning agents. *J Agric Food Chem* **48** 1393-1399

Kushido, N., Kabuyama, Y., Yamaguchi, O. and Homma, Y. (2001) Essential role for extracellular Ca^{2+} in JNK activation by mechanical stretch in bladder smooth muscle cells. *Am J Physiol Cell Physiol* **281** 1165-1172

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **277** 680-685

Laerke, P.E., Brierley, E.R. and Cobb, A.H. (2000) Impact-induced blackspots and membrane deterioration in potato (*Solanum tuberosum* L) tubers. *J Sci Food Agric* **80** 1332-1338

Lamb, C.J. and Dixon, R.A. (1997) The oxidative burst in plant disease resistance. *Annu Rev Plant Physiol Plant Mol Biol* **76** 419-422

Lander, H.M. (1997) An essential role for free radicals and derived species in signal transduction. *FASEB J* **11** 118-124

Langdon, T.T. (1987) Preventing of browning in freshly prepared potatoes without the use of sulfiting agents. *Food Technol* **41** 64-67

Lerner, A.B. (1953) Metabolism of phenylalanine and tyrosine. *Adv Enzymol* **14** 73-128

Lerner, A.B. and Fitzpatrick, T.B. (1950) Biochemistry of melanin formation. *Physiol Revs* **30** 91-126

Levine, R.L., Garland, D., Oliver, C.N., Amici, A., Climent, I., Lenz, A-G., Ahn, B-W., Shaltiel, S. and Stadtman, E.R. (1990) Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol* **186** 464-478

Levine, R.L., Williams, J.A., Stadtman, E.R. and Shacter, E. (1994) Carbonyl assays for determination of oxidatively modified proteins. *Methods Enzymol* **233** 346-357

Lin, K.T., Xue, J.Y., Sun, F.F. and Wong, P.Y. (1997) Reactive oxygen species participate in peroxynitrite-induced apoptosis in HL-60 cells. *Biochem Biophys Res Commun* **230** 115-119

Lindner, W.A., Hoffmann, C. and Grisebach, H. (1988) Rapid elicitor-induced chemiluminescence in soybean cell suspension cultures. *Phytochemistry* **27** 2501-2503

Lyras, L., Cairns, N.J., Jenner, A., Jenner, P. and Halliwell, B. (1997) An assessment of oxidative damage to proteins, lipids and DNA in brain from patients with Alzheimer's disease. *J Neurochem* **68** 2061-2069

McCord, J.M. (1974) Free radicals and inflammation: protection of synovial fluid by superoxide dismutase. *Science* **185** 529-531

McCord, J.M. (2000) The evolution of free radicals and oxidative stress. *Am J Med* **108** 652-659

McGurl, B., Orozco-Cardenas, M., Pearce, G. and Ryan, C.A. (1994) Overexpression of the prosystemin gene in transgenic tomato plants generates a systemic signal that constitutively induces proteinase-inhibitor synthesis. *Proc Natl Acad Sci USA* **91** 9799-9802

McNabnay, M., Dean, B.B., Bajema, R.W. and Hyde, G.M. (2000) The effect of potassium deficiency on chemical, biochemical and physical factors commonly associated with blackspot development in potato tubers. *Am J Potato Res* **76** 53-60

Mapson, L.W., Swain, T. and Tomalin, A.W. (1963) Influence of variety, cultural conditions and temperature of storage on enzymatic browning of tubers. *J Sci Food Agric* **14** 673-684

Mason, H.S. (1955) Comparative biochemistry of the phenolase complex. *Adv Enzymol* **16** 105-184

Massey, P.H., Thompson, H.C. and Smith, O. (1952) Varietal susceptibility of potatoes to internal blackspot. *Am Pot J* **29** 127-135

Matheis, G. and Belitz, H-D. (1978) Studies on enzymic browning of potatoes (*Solanum tuberosum* L) IV. Relationship between tyrosine turnover and rate of browning. *Z Lebensom Unters Forsch* **167** 97-100

Matheis, G. (1987a) Polyphenol oxidase and enzymatic browning of potatoes. II Enzymatic browning and potato constituents. *Chem Mikrobiol Technol Lebersm* **11** 33-41

Matheis, G. (1987b) Polyphenol oxidase and enzymatic browning of potatoes (S.tuberosum). Properties of potato polyphenol oxidase. *Chem Mikrobiol Technol Lebersm* **11** 5-12

Matheis, G. and Whitaker, J.R. (1984) Modification of proteins by polyphenol oxidase and peroxidase and their products. *J Food Biochem* **8** 137-162

Mathew, R. (1992) Impact bruise evaluation of Potato. *MSc thesis.* Agricultural Engineering Department, Washington State University

Mayer, A.M. (1987) Polyphenol oxidases in plants – recent progress. *Phytochem* **26** 11-20

Mayer, A.M. and Harel, E. (1979) Polyphenol oxidases in plants. *Phytochem* **18** 193-215

Mills, E.M., Gunasekar, P.G., Pavlakovic, G. and Isom, G.E. (1996) Cyanide-induced apoptosis and oxidative stress in differentiated PC12 cells. *J Neurochem* **67** 1039-1046

Mithofer, A., Daxberger, A., Fromhold-Treu, D. and Ebel, J. (1997) Involvement of an NADPH oxidase in the elicitor-inducible oxidative burst of soybean. *Phytochemistry* **45** 1101-1107

Molema, G.J., Klooster, J.J., Verwijs, B.R., Hendriks, M.M.W.B. and Breteler, H. (1997) Effect of impact body shape on subcutaneous tissue discolouration in potato tubers. *Neth J Agric Sci* **45** 407-421

Molnar-Perl, I. and Friedman, M. (1990a) Inhibition of food browning by sulfur amino acids. 2. Fruit juices and protein-containing foods. *J Agric Food Chem* **38** 1648-1651

Molnar-Perl, I. and Friedman, M. (1990b) Inhibition of food browning by sulfur amino acids. 3. Apples and potatoes. *J Agric Food Chem* **38** 1652-1656

Mondy, N.I. and Chaudra, S. (1981) Effect of zinc fertilization on yield, enzymatic discoloration, phenolic and nitrogenous constituents of potato tubers. *J Food Sci* **46** 1878-1880

Mondy, N.I., Klein, B.P. and Smith, L.I. (1960) The effect of maturity and storage on phenolic content, enzymatic activity and discoloration of potatoes. *Food Res* **25** 693-705

Mondy, N.I., Metcalf, C. and Plaisted, R.L. (1971) Potato flavour as related to chemical composition. 1. Polyphenols and ascorbic acid. *J Food Sci* **36** 459-464

Mondy, N.I., Mobley, O. and Gedde-Dahl, S.B. (1967) Influence of potassium fertilization on enzymatic activity, phenolic content and discoloration of potatoes. *J Food Sci* **32** 378-381

Mondy, N.I. and Munshi, C.B. (1993) Effect of maturity and storage on ascorbic acid and tyrosine concentrations and enzymatic discoloration of potatoes. *J Agric Food Chem* **41** 1868-1871

Morel, J-B. and Dangl, J.L. (1997) The hypersensitive response and induction of cell death in plants. *Cell Death Differ* **4** 671-683

Mosca, L., Blarzino, C., Coccia, R., Foppoli, C. and Rosei, M.A. (1998) Melanins from tetrahydroisoquinolines: spectroscopic characteristics, scavenging activity and redox transfer properties. *Free Radic Biol Med* **24** 161-167

Mulder, E.G. (1949) Mineral nutrition in relation to the biochemistry and physiology of potatoes. I. Effect of nitrogen, phosphate, potassium, magnesium and copper nutrition on the tyrosine content and tyrosinase activity with particular reference to blackening of the tubers. *Plant and Soil* **2** 59-121

Mulder, E.G. (1956) Effect of mineral nutrition of potato plants on the biochemistry and physiology of the tuber. *Neth J Agr Sci* **4** 333-356

Muneta, P. (1977) Enzymatic blackening in potatoes: influence of pH on dopachrome oxidation. *Am Pot J* **54** 387-393

Muneta, P.C. (1981) Comparison of inhibition of tyrosine oxidation in the enzymatic blackening of potatoes. *Am Pot J* **38** 1202-1204

Muneta, P.C. and Walradt, J. (1968) Cysteine inhibition of enzymatic blackening with polyphenol oxidase from potatoes. *J Food Sci* **33** 606-608

Murphy, T.M. and Auh, C-K. (1996) The superoxide synthases of plasma membrane preparations from cultured rose cells. *Plant Physiol* **110** 621-629

Narvaez-Vasquez, J., Orozco-Cardenas, M.L. and Ryan, C.A. (1994) A sulfhydryl reagent modulates systemic signalling for wound-induced and systemin-induced proteinase-inhibitor synthesis. *Plant Physiol* **105** 725-730

Newman, S.M., Eannetta, N.T., Yu, H., Prince, J.P., de Vicente, M.C., Tanksley, S.D. and Steffens, J.C. (1993) Organisation of the tomato polyphenol oxidase gene family. *Pl Mol Biol* **21** 1035-1051

Nordlund, J.J., Abdel-Malek, Z.A., Boissy, R.E. and Rheins, L.A. (1989) Pigment cell biology: an historical review. *J Invest Dermatol* suppl **92** 53s-60s

Omar, B.A. and McCord, J.M. (1991) Interstitial equilibration of superoxide dismutase correlates with its protective effect in the isolated rabbit heart. *J Mol Cell Cardiol* **23** 149-159

Oniki, T. and Takahama, U. (1992) Effect of redox reagents on ESR line shape of synthetic melanins. *Bull ChemSoc Jpn* **65** 6-13

Ophius, B.G., Hesen, J.C. and Kroesburgen, E. (1958) The influence of temperature during handling on the occurrence of blue discoloration inside potato tubers. *Eur Potato J* **1** 48-65

Orozco-Cardenas, M., McGurl, B. and Ryan, C.A. (1993) Expression of an antisense prosystemin gene in tomato plants reduces resistance toward *Manduca sexta* larvae. *Proc Natl Acad Sci USA* **90** 8273-8273

Palumbo, A., Di Cosmo, A., Gesualdo, F. and Hearing, V.J. (1997) Subcellular localisation and function of a melanogenic enzyme in the ink gland of *Sepia officinalis*. *Biochem J* **323** 749-756

Palumbo, A. and Jackson, I.J. (1995) Peroxidase activity in the ink gland of *Sepia officinalis* and pertial nucleotide sequence of a candidate small cDNA encoding the enzyme. *Biochim Biophys Acta* **1247** 173-178

Partington, J.C., Smith, C. and Bolwell, G.P. (1999) Changes in the location of polyphenol oxidase in potato *Solanum tuberosum* L. tuber during cell death in response to impact injury: comparison with wound tissue. *Planta* **207** 449-460

Passi, S. and Nazzaro-Porro, M. (1981) Molecular-basis of substrate and inhibitory specificity of tyrosinase- phenolic compounds. *Brit J Dermatol* **104** 659-665

Patil, S.S. and Zucker, M. (1965) Potato phenolases: purification and properties. *J Biol Chem* **240** 3938-3943

Pearce, G., Strydom, D., Johnson, S. and Ryan, C.A. (1991) A polypeptide from tomato leaves induces wound-inducible proteinase-inhibitor proteins. *Science* **253** 895-898

Peng, M. and Kuc, J. (1992) Peroxidase-generated hydrogen peroxide as a source of antifungal activity *in vitro* and on tobacco leaf disks. *Phytopathology* **82** 696-699

Percival, S.S. and Smith, K.R. (1994) Manganese stimulates the oxidative burst of differentiated HL-60 cells. *Biol Trace Element Res* **42** 243-252

Pierpoint, W.S. (1983) The major proteins in extracts of tobacco-leaves that are responding hypersensitively to virus-infection. *Phytochemistry* **22** 2691-2697

Prehn, J.H.M., Jordan, J., Ghadge, G.D., Preis, E., Galindo, M.F., Roos, R.P., Krieglstein, J. and Miller, R.J. (1997) Ca^{2+} and reactive oxygen species in staurosporine-induced neuronal apoptosis. *J Neurochem* **68** 1679-1685

Prota, G. (1992) Melanins and melanogenesis. San Diego *Academic Press*

Prota, G. (1997) What directions? *Pigment Cell Res* **10** 5-11

Qu, X., Kirschenbaum, L.J. and Borishet, E.T. (2000) Hydroxyterephthalate as a fluorescent probe for hydroxyl radicals: Application to hair melanin. *Phytochemistry Photobiology* **71** 307-313

Ragusa, R.J., Chow, C.K. and Porter, J.D. (1997) Oxidative stress as a potential pathogenic mechanism in an animal model of Duchenne muscular dystrophy. *Neuromuscul Disord* 7 379-386

Raper, H.S. and Wormall, A. (1923) The tyrosine-tyrosinase reaction. *Biochem J* **17** 454-469

Reeve, R.M., Timm, H. and Weaver, M.L. (1973) Cell wall thickness during growth of domestic and foreign potato cultivars. *Am Pot J* **50** 204-211

Reszka, K.J., Matuszak, Z. and Chignell, C.F. (1998) Lactoperoxidase-catalyzed oxidation of melanin by reactive nitrogen species derived from nitrite (NO_2^-): an EPR study. *Free Rad Biol Med* **25** 208-216

Reszka, J. and Jimbow, K. (1993) Electron donor and acceptor properties of melanin pigments in the skin. In Fuchs, J. and Packer, L. Eds, *Oxidative stress in dermatology.* New York: Marcel Dekker.

Reznick, A.Z. and Packer, L. (1994) Oxidative damage to proteins: spectrophotometric method for carbonyl assay. *Methods Enzymol* **233** 357-363

Rhodes, J.M.C. and Wooltorton, L.S.C. (1978) The biosynthesis of phenolic compounds in wounded plant storage tissues. In Kahl, G. Ed, *Biochemistry of wounded plant tissue.* Berlin : Walter de Gruyer

Riley, P.A. (1997) Melanin. *Intl J Biochem Cell Biol* **29** 1235-1239

Robinson, S.P. and Dry, I.B. (1992) Broad bean leaf polyphenol oxidase is a 60-kilodalton protein susceptible to proteolytic cleavage. *Plant Physiol* **99** 317-323

Ryan, C.A. (1990) Protease inhibitors in plants – genes for improving defenses against insects and pathogens. *Ann Rev Phytopathol* **28** 425-449

Sabba, R.P. and Dean, B.B. (1994) Sources of tyrosine in genotypes of *Solanum tuberosum* L. differing in capacity to produce melanin pigments. *J Amer Soc Hort Sci* **119** 770-774

Saitoh, M., Nishitoh, H., Fujii, M. (1998) Mammalian thioredoxin is a direct inhibitor of apoptosis signal- regulating kinase (ASK). *EMBO J* **17** 2596-2606

Sanchez-Ferrer, A., Laveda, F. and Garcia-Carmona, F. (1993) Partial purification of soluble potato polyphenol oxidase by partitioning in an aqueous two-phase system. *J Agric Food Chem* **41** 1219-1224

Sandstrom, P.A. and Buttke, T.M. (1993) Autocrine production of extracellular catalase prevents apoptosis of the human CEM T-cell line in serum-free medium. *Proc Natl Acad Sci USA* **90** 4708-4712

Sapers, G.M. (1993) Browning of foods: control by sulfites, antioxidants and other means. *Food Technol* **10** 75-84

Sapers, G.M., Douglas, F.W.Jnr., Bilyk, A., Hsu, A-F., Dower, H.W., Garzarella, L. and Kozempel, M. (1989) Enzymatic browning in Atlantic potatoes and related cultivars. *J Food Sci* **54** 362-365

Sapers, G.M. and Miller, R.L. (1992) Enzymatic browning control in potato with ascorbic acid-2-phosphates. *J Food Sci* **57** 1132-1135

Sapers, G.M. and Miller, R.L. (1995) Heated ascorbic acid / citric acid solution as browning inhibitors in pre-peeled potatoes. *J Food Sci* **60** 762-765

Sarna, T., Duleba, A., Korytowski, W. and Swartz, H.M. (1980) Interaction of melanin with oxygen. *Arch Biochem Biophys* **200** 140-148

Schaller, K. and Amberger, A. (1974) Zusammenhange zwischen clen fur die rohvefarbang der kartoffelknolle verantwortlishen inhaltsstoffen. *Qual Plant Plant Foods Hum Nutr* **24** 191

Schulze-Osthoff, K., Los, M. and Baeuerle, P.A. (1995) Redox signalling by transcription factors NF-kappa B and AP-1 in lymphocytes. *Biochem Pharmacol* **50** 735-741

Schulze-Osthoff, K., Bauer, M.K.A., Vogt, M. and Wesselborg, S. (1997) Oxidative stress and signal transduction. *Int J Vitam Nutr Res* **67** 336-342

Sealy, R.C. (1984) Radicals in melanin biochemistry. *Methods Enzymol* **105** 479-483

Sealy, R.C. (1985) Free radicals in melanin formation, structure and reactions. In Armstrong, D., Sohal, R.S., Cutler, R.G.C. and Slater, T.R. Eds. *Free radicals in molecular biology, aging, and disease.* New York: Raven Press.

Segal, A.W. and Abo, A. (1993) The biochemical basis of the NADPH oxidase of phagocytes. *Trends Biochem Sci* **18** 43-47

Sen, C.K. and Packer, L. (1996) Antioxidant and redox regulation of gene transcription. *FASEB J* **10** 709-720

Shahar, T., Hennig, N., Gutfinger, T., Hareven, D. and Lifschitz, E. (1992) The tomato 66.3kD polyphenoloxidase gene: molecular identification and developmental expression. *Plant Cell* **4** 135-147

Shatwell, K.P. and Segal, A.W. (1996) NADPH oxidase. *Intl J Biochem Cell Biol* **28** 1191-1195

Shibata, T., Prota, G. and Mishima, Y. (1993) Non-melanosoma regulatory factors in melanogenesis. *J Invest Dermatol* **100** 274S-280S

Silva, G.H., Chase, R.W., Hammerschmidt, R. and Cash, J.N. (1991) After-cooking darkening of Spartan Pearl potatoes as influenced by location, phenolic acids and citric acid. *J Agric Food Chem* **39** 871-873

Sinden, S.L., Deahl, K.L. and Aulenbach, B.B. (1976) Effect of glycoalkaloids and phenolics on potato flavor. *J Food Sci* **41** 520-523

Sommer, A., Ne'eman, E., Steffens, J.C., Mayer, A.M. and Harel, E. (1994) Import, targeting, and processing of a plant polyphenol oxidase. *Plant Physiol* **105** 1301-1311

Sowokinos, J.R. (2001) Biochemical and molecular control of cold-induced sweetening in potatoes. *Am J Pot Res* **78** 221-236

Spector, T. (1978) Refinement of the Coomassie blue method of protein quantitation. *Anal Biochem* **86** 142-145

Stadtman, E.R., Starke-Reed, P.E., Oliver, C.N., Carney, J.M. and Floyd, R.A. (1992) Protein modification in aging. *Exs* **62** 64-72

Stadtman, E.R., (1993) Oxidation of free amino acids and amino acid residues in prtoeins by radiolysis and by metal-catalyzed reactions *Annu Rev Biochem* **62** 797-821

Stark, J.C., Corsini, D.L., Hurley, P.J. and Dwelle, R.B. (1985) Biochemical characteristics of potato clones differing in blackspot susceptibility. *Am Pot J* **62** 657-666

Stevens, L.H. and Davelaar, E. (1996) Isolation and characterization of blackspot pigments from potato tubers. *Phytochemistry* **42** 941-947

Stevens, L.H. and Davelaar, E. (1997) Biochemical potential of potato tubers to synthesize blackspot pigments in relation to their actual blackspot susceptibility. *J Agric Food Chem* **45** 4221-4226

Stevens, L.H., Davelaar, E., Kolb, R.M., Pennings, E.J.M. and Smit, N.P.M. (1998) Tyrosine and cysteine are substrates for blackspot synthesis in potato. *Phytochemistry* **49** 703-707

Streller, S. and Wingsle, G. (1994) *Pinus sylvestris* L. needles contain extracellular Cu-Zn superoxide dismutase. *Planta* **192** 195-201

Sutherland, M.W. and Learmoth, B.A. (1997) The tetrazolium dyes MTS and XTT provide new quantitative assays for superoxide and superoxide dismutase. *Free Rad Res* **27** 283-289

Tenhaken, R., Levine, A., Brisson, L.F., Dixon, R.A. and Lamb, C.J. (1995) Function of the oxidative burst in hypersensitive disease resistance. *Proc Natl Acad Sci USA* **92** 4158-4163

Thygesen, P.W., Dry, I.B. and Robinson, S.P. (1995) Polyphenol oxidase in potato. A multigene family that exhibits differential expression patterns. *Plant Physiol* **109** 525-531

Timm, H., Yamaguchi, M., Hughes, D.L. and Weaver, M.L. (1976) Influence of ethylene on blackspot of potato tubers. *Am Pot J* **53** 49-56

Tobin, D. and Thody, A.J. (1994) The superoxide anion may mediate short- but not long-term effects of ultraviolet radiation on melanogenesis. *Exp Dermatol* **3** 99-105

Toshniwal, P.K. and Zarling, E.J. (1992) Evidence for increased lipid peroxidation in multiple sclerosis. *Neurochem Res* **17** 205-207

Tsukamoto, K., Palumbo, A., D'Ischia, M., Hearing, V.J. and Prota, G. (1992) 5,6-dihydroxyindole-2-carboxylic acid is incorporated in mammalian melanin. *Biochem J* **286** 491-495

Vaille, A., Jadot, G. and Eliza-Garay, A. (1990) Anti-inflammatory activity of various superoxide dismutases on polyarthritis in the lewis rat. *Biochem Pharmacol* **39** 247-255

Valverde, P., Manning, P., McNeil, C.J. and Thody, A.J. (1996) Activation of tyrosinase reduces the cytotoxic effects of the superoxide anion in B16 mouse melanoma cells. *Pigment Cell Res* **9** 77-84

Van Camp, W., Capiau, K., Van Montagu, M., Inze, D. and Slooten, L. (1996) Enhancement of oxidative stress tolerance in transgenic tobacco plants overproducing Fe-superoxide dismutase in chloroplasts. *Plant Physiol* **112** 1703-1714

Van Es, A. and Hartmans, K.J. (1975) Some differences in susceptibility to internal blackspot. *Potato Res* **18** 338

Vaughn, K.C., Lax, A.R. and Duke, S.O. (1988) Polyphenol oxidase: The chloroplast oxidase with no established function. *Physiol Plant* **72** 659-665

Verhaegen, S., McGowan, A.J., Brophy, A.R., Fernandes, R.S. and Cotter, T.G. (1995) Inhibition of apoptosis by antioxidants in the human HL-60 leukemia cell line. *Biochem Pharmacol* **50** 1021-1029

Vertregt, N. (1968) Relation between blackspot and composition of the potato tuber. *Eur Pot J* **11** 34-44

Wallaert, B., Aerts, C., Gressier, B., Gosset, P. and Voisin, C. (1993) Oxidative inactivation of alpha (1) – proteinase inhibitor by alveolar epithelial type II cells. *J Appl Physiol* **75** 2376-2382

Walker, J.R.L. (1977) Enzymic browning in foods: Its chemistry and control. *Food Technol N Zeal* **12** 19-25

Wientjes, F.B. and Segal, A.W. (1995) NADPH oxidase and the respiratory burst. *Sem Cell Biol* **6** 357-365

Willekens, H., Inze, D., Van Montagu, M. and Van Camp, W. (1995) Catalases in plants. *Mol Breed* **1** 207-228

Wojtaszek, P., Trethowan, J. and Bolwell, G.P. (1995) Specificity in the immobilization of cell-wall proteins in response to different elicitor molecules in suspension-cultured cells of French bean (*Phaseolus vulgaris* L). *Plant Mol Biol* **28** 1075-1087

Wojtaszek, P. (1997) Oxidative burst: An early plant response to pathogen infection. *Biochem J* **322** 681-692

Wood, J.M. and Schallreuter, K.U. (1991) Studies on the reactions between human tyrosinase, superoxide anion, hydrogen-peroxide and thiols. *Biochim Biophys Acta* **1074** 378-385

Wu, Q.Q. and Chen, Q. (2000) Mechanoregulation of chondrocyte proliferation, maturation and hypertrophy: Ion channel dependent transduction of matrix deformation signals. *Exp Cell Res* **256** 383-391

Yahruas, T., Chandra, S., Legendre, L. and Low, P.S. (1995) Evidence for a mechanically induced oxidative burst. *Plant Physiol* **109** 1259-1266

Yu, H., Newman, S.M., Eanetta, N.T., Fischer, M.L. and Steffens, J.C. (1992) Cloning of tomato polyphenol oxidase cDNAs and enzyme conservation between polyphenol oxidases and tyrosinases. *Plant Physiol* **96s** 5

Zettl, U.K., Mix, E., Zielasek, J., Stangel, M., Hartung, H.P. and Gold, R. (1997) Apoptosis of myelin-reactive T cells induced by reactive oxygen and nitrogen intermediates *in vitro*. *Cell Immunol* **178** 1-8

Zitnac, A. and Filadelfi, M.A. (1985) Estimation of taste thresholds of three potato glycoalkaloids. *J Can Inst Food Sci Technol* **18** 337-339

Zughaier, S.M., Ryley, H.C. and Jackson, S.K. (1999) A melanin pigment purified from an epidemic strain of *Burkholderia cepacia* attenuates monocyte respiratory burst activity by scavenging superoxide anion. *Infection Immunity* **67** 908-913

APPENDIX ONE

Growing conditions for potato tubers

PHYSIOLOGICAL AND BIOCHEMICAL ASPECTS OF BRUISING IN POTATO TUBERS

XAAKC/A ADAS STUDY REVIEW 1997

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XAAKC/A ADAS STUDY REVIEW

PHYSIOLOGICAL AND BIOCHEMICAL ASPECTS OF BRUISING IN POTATO TUBERS

Contract Manager and Study Director: (author of report)

Dr J J J Wiltshire
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Status of work: Concluded

Year of experiment: 2 of 2

Period covered: 1996/97

Electronic location of report:

Terrington LAN fileserver,
Filename:
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(This location will change when the file is archived)

ABSTRACT

Potatoes were grown under carefully monitored field conditions to provide material for biochemical studies at The Nottingham Trent University.

This report summarises the field and husbandry conditions under which the crops were grown at ADAS Arthur Rickwood and ADAS Terrington. The R & D Protocol is appended (Appendix 1), as are the site reports (Appendices 2 and 3).

OBJECTIVES

Overall Objective

To provide practical guidance to the potato industry on how best to reduce commercial losses due to bruising by developing an improved understanding of the biochemical and physiological processes involved in the bruising reaction.

Specific Objective

To provide tubers grown and harvested under closely controlled conditions to a specification agreed by collaborators at The Nottingham Trent University for the purposes of a biochemical study.

MATERIALS AND METHODS

Full details are given in the R & D Protocol (Appendix 1).

There were two sites (ADAS Arthur Rickwood and ADAS Terrington) and six treatments. The treatments were: three cultivars (Maris Piper, Pentland Dell and Record) and two physiological ages for each (0 day-degrees and 500 day-degrees). Physiological age treatments were applied at ADAS Terrington, using a base temperature of 4°C.

Seed potatoes were hand planted, grown and hand harvested under conditions of normal crop husbandry for the site. All aspects of crop management were carefully recorded. Crop management details for ADAS Arthur Rickwood and ADAS Terrington are given in Appendices 2 and 3 respectively.

At harvest tubers were lifted with garden forks, and picked directly into trays for storage at Sutton Bridge Experimental Station. The trays were transported to Sutton Bridge on the harvest days.

Meteorological data were recorded at the Meteorological Sites at ADAS Arthur Rickwood and ADAS Terrington.

RESULTS

Crop husbandry records are presented in Appendices 2 and 3. These include monthly rainfall data and temperatures at harvest.

CONCLUSIONS

Conclusions cannot be drawn from the work reported here in isolation from the biochemical experiments which are ongoing.

SITE LOCATIONS AND REPORT AUTHORS

Site Code	Site location	Site Report Author
1	ADAS Arthur Rickwood	Mr J Mottram
2	ADAS Terrington	Dr J J J Wiltshire

APPENDIX 1

R & D PROTOCOL

1. TITLE

Physiological and biochemical aspects of bruising in potato tubers

2. REFERENCE NUMBER

XAAKC/A

3. STUDY DIRECTOR

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4. CUSTOMER

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Contact:

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5. CONTRACT MANAGER

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6. SITES AND SITE MANAGERS

Site 1: ADAS Arthur Rickwood
Site Manager: Mr P Saunders, ADAS Arthur Rickwood, Mepal, Ely, Cambs, CB6 2BA
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Site 2: ADAS Terrington
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7. OBJECTIVES

Specific Objective
To provide tubers grown and harvested under closely controlled conditions to a specification agreed by collaborators at The Nottingham Trent University for the purposes of a biochemical study.

ADAS will also assist Nottingham Trent University in the preparation of guidance document for the industry, on how best to reduce commercial losses due to bruising. This will contribute towards the attainment of the overall objective, which is: to provide practical guidance to the potato industry on how best to reduce commercial losses due to bruising by developing an improved understanding of the biochemical and physiological processes involved in the bruising reaction. This aspect of the contract is not covered by this protocol.

8. TIMETABLE

8.1. Duration Three years, October 1995 to September 1998.

8.2. Milestones

(i)	Harvest tubers by hand from commercial fields of Pentland Dell and Record, and transport to Sutton Bridge Experimental Station for storage.	October 1995
(ii)	Establish field plots at both sites.	April 1996
(iii)	Hand harvest tubers from field plots and transport to Sutton Bridge Experimental Station for storage.	October 1996
(iv)	Submit annual report to the customer.	December 1996

(v)	Establish field plots at both sites.	April 1997
(vi)	Hand harvest tubers from field plots and transport to Sutton Bridge Experimental Station for storage.	October 1997
(vii)	Submit annual report to the customer.	December 1997
(viii)	Assist the customer in preparation of guidance document for the industry.	August 1998
(ix)	Hold meeting to promote technological transfer of the project findings.	September 1998

9. TREATMENTS

Treatments will be as agreed with the lead contractor, The Nottingham Trent University, and are likely to change as the study progresses. For this reason, the treatments will be specified in protocol amendments.
The following factors will be addressed:

- ◊ cultivar,
- ◊ physiological age,
- ◊ harvesting procedure.

10. EXPERIMENTAL DESIGN AND ANALYSIS

10.1. Design

Unreplicated

10.2. Analysis

None, since the purpose is to produce tubers under a range of closely controlled conditions for a biochemical study. Statistical analysis of the results of the biochemical experiments will be facilitated by replicating those experiments by taking from the store randomly selected tubers of each treatment. The analysis relating to the biochemical experiments will be done by staff at The Nottingham Trent University, as directed by their own statisticians.

11. MATERIALS

The materials listed here are additional to those materials specified in the SOPs referred to in this document.

- ◊ Seed potatoes of the appropriate cultivars (Section 9)
- ◊ Trays (supplied by Sutton Bridge Experimental Station) for storage of harvested tubers
- ◊ SOPs listed in Section 17

12. METHODS

12.1. Apply seed treatments at ADAS Terrington. When the appropriate physiological age (Section 9) has been achieved store the seed tubers at 2°C (except during transport, see Section 12.2.) until 4 days before planting, when the temperature will be raised to ambient..

12.2. When the appropriate physiological age has been achieved transport half of the seed tubers to ADAS Arthur Rickwood.

12.3. Draw up a plan of the experiment (SOP AGRON/002). Plot size will be specified in a protocol amendment, and will be as agreed with the lead contractor, The Nottingham Trent University.

12.4. Mark out the experiment plots (SOP AGRON/017).

12.5. Hand-plant the seed tubers (SOP POTS/034) within the normal planting period for the local farm crop.

12.6. Mark plots with numbered plot markers.

12.7. Fertilisers, other agrochemicals and irrigation should be applied according to the normal agronomic practice for the farm crop, during crop growth and for haulm destruction prior to harvest.

12.8. Hand harvest (SOP POTS/039) tubers on dates which will be specified in a protocol amendment as agreed with the lead contractor, The Nottingham Trent University. Place the tubers directly into numbered trays for transport to Sutton Bridge Experimental Station.

12.9. Transport the harvested tubers on the day of harvest to Sutton Bridge Experimental Station for storage.

13. ASSESSMENTS AND RECORDS

13.1. Meteorological data

Take daily measurements of the following data from the closest recording station:

- ◊ air temperature - min, max, mean;
- ◊ soil temperature - surface, 10 cm, 30 cm;
- ◊ rainfall;
- ◊ accumulated solar radiation.

13.2. Keep a detailed diary of all field operations, including cultivations prior to planting, and all agrochemical applications.

13.3. Record date of 50% emergence.

13.4. Record date of canopy closure.

14. DATA HANDLING

14.1. File records

All paper records should be kept as detailed in SOP DATA/020.

14.2. Collection

All data will be collected manually except for meteorological data which may be collected electronically.

14.3. Collation and Analysis

Meteorological data will be collated using Excel (SOP DATA/033). Other data will be mainly descriptive (e.g. husbandry practices) and will be collated in the site reports using WORD. The analysis of data from the biochemical experiments will be done by staff at The Nottingham Trent University, as directed by their own statisticians.

15. REPORTS

Site reports will be produced by 30 November in each year when tubers are produced (SOP ADMIN/008). An annual report will be sent to the customer (The Nottingham Trent University) before the end of each calendar year.

Reports to the funding body (MAFF) are the responsibility of the lead contractor, The Nottingham Trent University.

16. ARCHIVING

All data and documents relating to this experiment will be archived at ADAS Rosemaund (SOPs DATA/014, DATA/036).

17. SOP LIST

ADMIN/008	The production of R&D reports
AGRON/002	Preparing experiment site plans
AGRON/017	Marking out experiment plots
DATA/014	Preparing and archiving of study specific raw data packages
DATA/020	Guidelines for keeping manual records of experiments
DATA/033	Collating experimental data using Excel for Windows
DATA/036	Preparation of computer based material for archiving
POTS/034	Hand planting potatoes
POTS/039	Harvesting potato plots

The instructions in these SOPs must be followed precisely unless a specific variation to a cited SOP is demanded in this protocol.

18. AUTHORISATION

	NAME	SIGNATURE	DATE
Contract manager, Study director and Site Manager, Site 2	Dr J J J Wiltshire		
Site Manager, Site 1	Mr P Saunders		

19. DISTRIBUTION

Contract manager, Study director and Site Manager, Site 2	Dr J J J Wiltshire
Site Manager, Site 1	Mr P Saunders
Account manager for MAFF HPD division	Dr R Bailey
Representatives of the customer	Professor A H Cobb
	Dr E R Brierley
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